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13. ABSTRACT *(Maximum 200 Words)*

The insulin-like growth factor I receptor (IGF-IR) is a multifunctional tyrosine kinase that has been implicated in breast cancer. The IGF-IR is often overexpressed in ER-positive breast tumors and this feature predicts tumor drug- and radio-resistance and cancer recurrence at the primary site.

Over the period 1996-2000, we studied IGF-IR signaling pathways that may contribute to breast cancer development and progression. We developed different cell lines with either overexpression or downregulation of the IGF-IR or its major signaling substrates. Using these model systems, we characterized cross-talk between estrogen receptor and IGF-IR signaling, and we provided the first evidence that the IGF-IR, especially the IGF-IR/IRS-1/PI-3K pathway, plays a major role in 2 processes involved in cancer progression: antiestrogen-resistance and estrogen-independence.

We also described, for the first time, interactions between the IGF-IR and cell-cell (cadherins and catenins) and cell-substrate (integrins) systems. Our data indicated that the IGF-I signal can increase cell adhesion and motility, the functions that are critical for metastatic cell spread. Recently, we demonstrated that in ER-negative metastatic cells, IGF-I-induced motility is mediated through PI-3 and p38 kinases, and is inhibited by the activation of ERK1/ERK2 kinases.

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Appendix : Manuscripts:

1. Bartucci, M., Morelli, C., Mauro, L., Ando', S., Surmacz, E. IGF-I receptor signaling and function are different in non-metastatic and metastatic breast cancer cells, 2000, submitted
2. Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.
3. Mauro, L., Sisci, D., Bartucci, M., Salerno, M., Kim, J., Tam, T., Guvakova, M., Ando, S., Surmacz, E. SHC-alpha5 beta1 integrin interactions regulate breast cancer cell adhesion and motility. Exp. Cell Res., 252: 439-448, 1999.
4. Guvakova, M., Surmacz, E. The activated insulin-like growth factor I receptor induces depolarization in breast epithelial cells characterized by actin filament disassembly and tyrosine dephosphorylation of FAK, Cas, and paxillin. Exp. Cell Res., 251: 244-255, 1999.
5. Salerno, M., Sisci, D., Mauro, L., Guvakova, M., Ando, S., Surmacz, E. Insulin receptor substrate 1 (IRS-1) is a target of a pure antiestrogen ICI 182,780 in breast cancer cells. Int. J. Cancer, 81: 299-304, 1999.
6. Ando, S., Panno, M. L., Salerno, M., Sisci, D., Mauro, L., Lanzino, M., Surmacz, E. Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. Biochem. Biophys. Res. Com., 253: 315-319, 1998.
7. Surmacz, E., Guvakova, M., Nolan, M., Nicosia, R., Sciacca, L. Type I insulin-like growth factor receptor function in breast cancer. Breast Cancer Res. Treat. 47: 255-267, 1998.
8. Guvakova, M.A., Surmacz, E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. Cancer Res. 57: 2606-2610, 1997.
9. Nolan, M. K., Jankowska, L., Prisco, M., Xu, S., Guvakova, M. A., Surmacz, E. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. Int. J. Cancer 72: 828-834, 1997.
10. Guvakova, M.A. Surmacz, E. Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival and promote E-cadherin-mediated cell-cell adhesion in human breast cancer cells. Exp. Cell Res. 231: 149-162, 1997.

INTRODUCTION

Insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a ubiquitous multifuctional tyrosine kinase. The IGF-IR regulates normal breast development; however, hyperactivation of the same receptor has been implicated in breast cancer. In particular, overexpression of either the IGF-IR or its major signaling substrate IRS-1 in estrogen receptor (ER)-positive breast tumors has been linked with cancer recurrence at the primary site. Furthermore high circulating levels of IGF-I (an IGF-IR ligand) have been associated with increased breast cancer risk in premenopausal women (1).

Although current evidence suggests that abnormal activation of the IGF-IR may contribute to the autonomous growth and increased survival of ER-positive breast cancer cells at the primary site, the function of this receptor in breast cancer metastasis is not clear. For instance, some small clinical studies demonstrated a correlation between IGF-IR expression in node-positive tumors and worse prognosis. Other data linked IGF-IR expression with better clinical outcome as the IGF-IR was predominantly expressed in a subset of breast tumors with good prognostic characteristics. In the experimental setting, IGF-I is not a mitogen for ER-negative metastatic cells, but acts as a chemoattractant for these cells, which may suggest a role of the IGF-IR in cell spread (1).

Using in vitro model systems developed in our laboratory, we asked how the IGF-IR and its different signaling pathways contribute to breast tumor development and progression. We focused on abnormal proliferation and survival, enhanced resistance to anti-tumor treatments, and augmented migration and invasion. We also addressed the differential IGF-I response in ER-positive and ER-negative cells.

TECHNICAL REPORT

The experiments proceeded according to the modified and approved SOW.

In Year 1, we developed and characterized several MCF-7 cell lines expressing different levels of the IGF-IR and we characterized their estrogen and IGF-I growth requirements. We also studied invasive properties in these cells. The results demonstrated that high levels of the IGF-IR improve IGF-I response and significantly reduce estrogen growth requirements. Overexpression of the IGF-IR was also associated with an increased cell aggregation (mediated by E-cadherin) and improved survival of breast cancer cells in 3-D culture.
Reported in: Guvakova, M.A. and Surmacz, E. Exp. Cell Res. 231: 149-162, 1997 and Surmacz, E. et al., Breast Cancer Res. Treat. 47: 255-267, 1998. (Appendix).

In Year 2, we begun to study the interactions between estrogen and IGF-IR signaling systems in breast cancer cells. These experiments continued throughout the Years 3 and 4. We found that both pure and non-steroid antiestrogens (ICI 182,780 and Tamoxifen, respectively) downregulate IGF-I signaling in ER-positive cells. The action of antiestrogens was associated with downregulation of IGF-IR tyrosine phosphorylation as well as decreased expression of IRS-1 and subsequent attenuation of IRS-1/PI-3K signaling. In agreement with these observations, we documented, in collaboration with the Ando' group (University of Calabria, Cosenza, Italy), that estrogens upregulate IRS-1 expression and induce IRS-1/PI-3K signaling. We also reported that overexpression of IRS-1 (but not the second major IGF-IR substrate,

SHC) induces antiestrogen resistance in breast cancer cells. Cumulatively, the results suggested that hyperactivation of the IRS-1 pathway may be an important factor interfering with endocrine therapy in ER-positive tumors. *Reported in: Guvakova, M.A., and Surmacz, E. Cancer Res. 57: 2606-2610, 1997; Salerno, M. et al., Int. J. Cancer, 81: 299-304, 1999; Ando, S. et al.,. Biophys. Res. Com., 253: 315-319, 1998 (Appendix); and Salerno, M. et al., 2000. Submitted.*

In Years 3 and 4, we addressed the importance of the IGF-IR in cell-matrix adhesion and motility, the processes that are critical for metastatic cell spread. We found that IGF-I regulates cell motility in ER-positive and ER-negative breast cancer cells. IGF-I effects were associated with actin cytoskeleton disassembly and tyrosine dephosphorylation of FAK (focal adhesion kinase), paxillin, and p130 Cas. In addition, IGF-I-dependent cell migration depended on PI-3 and p38 kinases and was inhibited by ERK1/ERK2 kinases. Using cell lines with either overexpression or downregulation of SHC, we found that this IGF-IR substrate is involved in the interactions with alpha5 beta1 integrin and modulates cell adhesion and motility on fibronectin. *Reported in: Mauro, L. et al., Exp. Cell Res., 252: 439-448, 1999; Guvakova, M.A., and Surmacz, E. Exp. Cell Res., 251: 244-255, 1999; and Bartucci, M. et al., 2000. Submitted. (Appendix).*

The studies on IGF-IR-induced motility and invasion will be continued with cell lines expressing IGF-IR mutants (described in 1999 Annual Report) and IRS-1 mutants (not yet developed).

The status of current knowledge on IGF-IR signaling in breast cancer has been summarized in 2 invited peer-reviewed review articles: *Surmacz, E. et al. Breast Cancer Res. Treat. 47: 255-267, 1998; and Surmacz, E. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.(Appendix).*

Key Research Accomplishments:

- Demonstrated, for the first time, that high levels of the IGF-IR or IRS-1, but not SHC, induce estrogen-independence in ER-positive cells;
- Demonstrated that estrogen stimulates the expression of IRS-1, whereas antiestrogens downregulate IGF-IR/IRS-1/PI-3K signaling in ER-positive cells;
- Established that overexpression of the IGF-IR provides survival signals in 3-D culture;
- Demonstrated that the IGF-IR increases cell-cell adhesion and co-associates with adhesion proteins E-cadherin, alpha-, and beta-catenins;
- Determined that SHC is necessary for IGF-I-dependent cell migration and SHC/alpha5 beta1 integrin (fibronectin receptor) interactions regulate breast cancer cell adhesion and motility;
- Demonstrated that IGF-IR transmits different signals in ER-positive and ER-negative cells. In ER-positive non-metastatic cells, the IGF-IR regulates growth, survival, and migration, whereas in ER-negative metastatic cells, its mitogenic functions are attenuated but motogenic signaling is still operative;
- Determined that IGF-I-dependent migration in both cell types is mediated through p38 kinase and PI-3K pathways, and is inhibited by ERK1/ERK2 pathways.

Reportable Outcomes:

1. Manuscripts, abstracts and scientific presentations:

Manuscripts:

1. Bartucci, M., Morelli, C., Mauro, L., Ando', S., Surmacz, E. IGF-I receptor signaling and function are different in non-metastatic and metastatic breast cancer cells. 2000. Submitted.
2. Salerno, M., Mauro, L., Bellizzi, D., Sisci, D., Panno, M-L., Surmacz, E., Ando', S. Upregulation of IRS-1 expression by estradiol amplifies insulin signaling in human breast cancer cells.2000. Submitted.
3. Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.
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11. Guvakova, M.A. Surmacz, E. Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival and promote E-cadherin-mediated cell-cell adhesion in human breast cancer cells. Exp. Cell Res. 231: 149-162, 1997.

Presentations/Abstracts:

1. Morelli, C., Bartucci, M., Mauro, L., Ando' S., Surmacz, E. Insulin-like growth factor I receptor (IGF-IR) signaling in metastatic breast cancer cells. The Endocrine Society Annual Meeting, Toronto, Canada, June 21-24, 2000.
2. Surmacz, E. Insulin-like growth factor I signaling is regulated by estrogen receptor in breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.

3. Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.
4. Bartucci, M., Mauro, L., Salerno, M., Morelli, C., Ando', S., Surmacz, E. Function of the insulin-like growth factor I receptor in metastatic breast cancer cells. 22nd Annual Breast Cancer Symposium. San Antonio, TX, December 8-11, 1999
5. Surmacz, E, Mauro, L., Ando', S. New insights into IGF-I-dependent regulation of cell-cell adhesion in breast cancer cells. 5th International Symposium on IGFs. Brighton, U.K., October 31-November 4, 1999
6. Guvakova, M., Surmacz, E. Tyrosine kinase activity of the IGF-IR is required for the development of breast cancer cell aggregates in three-dimensional culture. AACR Annual Meeting, Philadelphia, PA, April 10-14, 1999
7. Guvakova, M., Surmacz, E. IGF-IR tyrosine kinase is required for breast cancer cell motility. Specificity in Signal Transduction. Keystone Symp.Keystone, CO, April 9-14, 1999
8. Mauro, L., Sisci, D., Salerno, M., Ando', S., Surmacz, E. Role of SHC signaling in breast cancer cell adhesion and motility. 21st Annual Breast Cancer Symposium, San Antonio, TX, December 12-15, 1998
9. Guvakova, M., Surmacz, E IGF-IR stimulates breast epithelial cell motility via reorganization of the actin cytoskeleton, remodeling of focal contacts, and modulation of the phosphorylation status of focal adhesion proteins: FAK, Cas, and paxillin.14th Annual Symposium on Cellular Endocrinology, Lake Placid, NY, September 24-27, 1998
10. Guvakova, M., Surmacz, E. IGF-IR modulates breast cancer cell motility through the regulation of p125 FAK/p130 CAS. 37th American Society for Cell Biology Annual Meeting, Washington D.C., December 13-17, 1997
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15. Ando, S., Salerno, M., Sisci, D., Panno, M. L., Nolan, M. K., Surmacz, E. IRS-1 signaling and insulin-induced modulation of estrogen receptors in breast cancer. The Endocrine Society 79th Annual Meeting, Minneapolis, June 11-14, 1997
16. Guvakova, M., Surmacz, E. Tamoxifen differentially modulates IGF-IR signaling pathways in breast cancer cells. The Endocrine Society 79th Annual Meeting, Minneapolis, June 11-14, 1997
17. Guvakova, M., Surmacz, E. Overexpression of the IGF-IR, but not of its substrate IRS-1, stimulates E-cadherin-mediated cell-cell adhesion in human breast cancer cells. 49th Annual

Symposium on Fundamental Cancer Research "Regulatory mechanisms in growth and differentiation", Houston, TX, October 22-25, 1996.

18. Guvakova, M., Surmacz, E. Overexpressed IGF-I receptors induce mitogenesis, enhance survival and promote E-cadherin-mediated cell-cell adhesion in human breast cancer. 10th International Congress of Endocrinology, San Francisco, June 12-15, 1996.
19. Guvakova, M., Surmacz, E. Biological effects of the overexpression of the insulin-like growth factor I receptor (IGF-IR) in human breast cancer cells. Annual AACR Meeting, Washington D.C., April 20-24, 1996.
20. Nolan, M., Jankowska, Prisco, M., Guvakova, M., Surmacz, E. Differential roles of IRS-1 and SHC signaling pathways in human breast cancer cells. Annual AACR Meeting, Washington D.C., April 20-24, 1996.

Talks

1. Surmacz E. Is IGF-IR involved in breast cancer? Lankenau Cancer Research Center, Wynnewood, PA, February 3, 2000.
2. Surmacz E. Diabetes, insulin treatment and breast cancer. Novo Nordisk Pharmaceuticals. Princeton, NJ., January 25, 2000.
3. Surmacz, E. IGF-IR and breast cancer. Department of Cellular Biology, University of Calabria, Cosenza, Italy, December 9, 1999.
4. Surmacz, E. IGF-IR signaling in breast cancer. University of Gent, Gent, Belgium, September 29, 1999.
5. Surmacz, E. Is IGF-I receptor involved in breast cancer? Staff Seminar Series, Kimmel Cancer Institute, Philadelphia, PA, January 18, 1999
6. Surmacz, E. IGF and breast cancer. Prolactin and Growth Hormone: Mechanism and Function International Hannah Symposium, Ayr, Scotland, UK, September 9-11, 1998.
7. Surmacz, E. IGF-IR and breast cancer. Department of Medical Genetics, University of Ferrara, Italy, May 13, 1998.
8. Surmacz, E. IGF-IR signaling in breast cancer. Oncology Clinic, University of Modena, Italy, May 12, 1998.
9. Surmacz, E. IGF-IR signaling in breast cancer. Department of Pathology, Allegheny University, Philadelphia, PA, December 16, 1997.
10. Surmacz, E. IGF-IR in breast cancer. Department of Pathology, University of Catania, Italy, May 6, 1997.
11. Surmacz, E. Mitogenic and transforming signaling of the IGF-IR. Department of Endocrinology, University of Catania, Italy, May 5, 1997.
12. Surmacz, E. IGF-I receptor signaling in breast cancer. Symposium "Endocrine and hormone-dependent tumors", University of Calabria, Cosenza, Italy, May 28, 1996.

2. Patents and licences: None

3. Degrees: N/A

4. Development of biologic reagents:

Several new cell lines have been generated and characterized:

- MCF-7 cells overexpressing the IGF-IR on the level 500,000-3,000,000 receptors/cell (clones 12, 34, 15 and 17);
- MCF-7 cells expressing dominant negative mutants of the IGF-IR: MCF-7/KR and MCF-7/KA;
- MCF-7 cells overexpressing signaling molecules SHC or IRS-1, MCF-7/SHC;
- MCF-7 cells expressing antisense RNA to IRS-1 or SHC;
- metastatic breast cancer cell lines overexpressing IGF-IR, MDA-MB-231/IGF-IR clones 2 and 31.

5. Databases: None

6. Funding applied for:

- DOD Breast Cancer Research Program. "IGF-IR, Cell-cell Adhesion and Metastasis" Idea Award, 1999-2002. E. Surmacz, P.I., Funded.
- Italian-American Cancer Foundation, "Evolution of IGF-IR signaling during breast cancer progression", 2000/2001, Special Fellowship for Dr. Catia Morelli, E. Surmacz, Sponsor. Funded.
- UICC International Union Against Cancer, "IGF-IR-mediated cell-cell adhesion", Short-term Fellowship for Dr. Loredana Mauro, E. Surmacz, Program Director, 1999. Funded.
- UICC International Union Against Cancer, "IGF-I-dependent Antiestrogen Resistance in 3-D Culture", Short-term Fellowship for Dr. Michele Salerno, E. Surmacz, Program Director, 1998. Funded.
- DOD Breast Cancer Research Program. "Cell-cell adhesion and the IGF-IR in breast cancer", Postdoctoral Fellowship for M. Guvakova, E. Surmacz, Sponsor. 1997-2000. Funded.
- NIHRO1 "IGF-IR in breast cancer", E. Surmacz, P.I., 2000-2003 competitive continuation, Pending.
- Susan G. Komen Breast Cancer Foundation "Evolution of IGF-I signaling in breast cancer progression", E. Surmacz, P.I., 2001-2003, Pending.
- DOD Breast Cancer Research Program, "Leptin-obesity protein and breast cancer metastasis" Concept Proposal, E. Surmacz, P.I., 2000/2001, Pending.

7. Employment applied for: None

8. Research personnel supported by this award: this CDA supported 50% of the P.I. salary only.

CONCLUSIONS

Our research determined that the IGF-IR, especially the IGF-IR/IRS-1 pathwayshould be considered a possible target for anti-breast cancer therapy.

REFERENCES

1. Surmacz, E. Function of the IGF-IR in breast cancer. J. Mammary Gland Biol. Neopl., 5: 95-105, 2000.

IGF-I Receptor Signaling and Function are Different in Non-Metastatic and Metastatic Breast Cancer Cells

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Running title: IGF-IR in metastatic breast cancer

Key words: breast cancer, IGF-I receptor, metastasis, migration, estrogen receptor

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ABSTRACT

The insulin-like growth factor receptor (IGF-IR) is a ubiquitous and multifunctinal tyrosine kinase that has been implicated in breast cancer development. In ER-positive breast tumors, the levels of IGF-IR and its substrate IRS-1 are often elevated and these characteristics have been linked with increased radio-resistance and cancer recurrence. *In vitro*, activation of the IGF-IR/IRS-1 pathway in ER-positive cells improves growth and counteracts apoptosis induced by anticancer treatments.

The role of the IGF-IR in ER-negative metastatic breast cancer is not clear. Highly aggressive, ER-negative breast cancer cell lines express low levels of the IGF-IR and fail to respond to IGF-I with mitogenesis. On the other hand, inhibition of the IGF-IR reduces metastatic potential of these cells, suggesting a role of this receptor in late stages of the disease. Here we examined IGF-IR signaling and function in ER-negative metastatic breast cancer cells. Using MDA-MB-231 cells and their IGF-IR-overexpressing derivatives, we demonstrated that IGF-I acts as a chemoattractant for these cells. The extent of IGF-I-induced migration reflected IGF-IR levels and required the activation of PI-3K and p38 kinases. The same pathways promoted IGF-I-dependent motility in ER-positive MCF-7 cells.

In contrast with the positive effects on cell migration, IGF-I was unable to stimulate the growth or improve survival in MDA-MB-231 cells, while it induced mitogenic and anti-apoptotic effects in MCF-7 cells. Moreover, IGF-I counteracted the action of PI-3K and ERK1/ERK2 inhibitors in MCF-7 cells, while it had no protective effects in MDA-MB-231 cells. The impaired IGF-I growth response in ER-negative cells was not caused by the low IGF-IR expression, defective IGF-IR tyrosine phosphorylation, or improper tyrosine phosphorylation of IRS-1. Also, the acute (15 min) IGF-I activation of PI-3 and Akt kinases was similar in ER-negative and ER-positive cells. However, a long-term (2 days) IGF-I exposure induced the PI-3K/Akt pathway only in MCF-7 cells. The reactivation of this pathway in ER-negative cells by overexpression of constitutively active Akt mutants was not sufficient to improve proliferation or survival (with or without IGF-I), which indicated that other pathways are also required to support these functions.

Our results suggest that IGF-IR function undergoes evolution during breast cancer progression from the ER-positive to ER-negative phenotype: growth-related signaling becomes attenuated, while non-mitogenic processes, such as migration, still remain under IGF-IR control.

INTRODUCTION

The insulin-like growth factor I (IGF-I) receptor (IGF-IR) is a ubiquitous, transmembrane tyrosine kinase that has been implicated in different growth-related and – unrelated processes critical for the development and progression of malignant tumors, such as proliferation, survival, and anchorage-independent growth as well as cell adhesion, migration, and invasion (1, 2).

The IGF-IR is necessary for normal breast biology, but recent clinical and experimental data strongly suggest that the same receptor is involved in the development of breast cancer (1, 3). The IGF-IR is overexpressed (up to 14-fold) in estrogen receptor (ER)-positive breast cancer cells compared with its levels in normal epithelial cells (1, 4, 5). The elevated expression and hyperactivation of the IGF-IR has been linked with increased radio-resistance and cancer recurrence at the primary site (4). Similarly, high levels of insulin-receptor substrate 1 (IRS-1), a major signaling molecule of the IGF-IR, correlated with tumor size and shorter disease-free survival in ER-positive tumors (6, 7).

IGF-IR ligands, IGF-I and IGF-II, are strong mitogens for many hormone-dependent breast cancer cell lines and have been found in the epithelial and/or stromal component of breast tumors (1). Importantly, higher levels of circulating IGF-I predict increased breast cancer risk in premenopausal women (8). *In vitro*, activation of the IGF-IR, especially the IGF-IR/IRS-1/PI-3K pathway in ER-positive breast cancer cells, counteracts apoptosis induced by different anti-cancer treatments or low concentrations of hormones (1, 9-11). On the other hand, overexpression of either the IGF-IR or IRS-1 in ER-positive breast cancer cells improves responsiveness to IGF and, in consequence,

results in estrogen-independent proliferation (1, 12, 13). In agreement with these observations, blockade of IGF-IR activity with various reagents targeting the IGF-IR or its signaling through IRS-1/PI-3K reduced the growth of breast cancer cells *in vitro* and/or *in vivo* (1, 12, 14-17).

The requirement for the IGF-IR/IRS-1 pathway for growth and survival appears to be a characteristic of ER-positive, more differentiated, breast cancer cells. By contrast, ER-negative tumors and cell lines, which frequently exhibit a less differentiated, mesenchymal phenotype, express low levels of the IGF-IR and often decreased levels of IRS-1 (1, 17). Notably, these cells do not respond to IGF-I with growth (1, 18-21). Despite the lack of IGF-I mitogenic response, metastatic potential of ER-negative breast cancer cells can be effectively inhibited by different compounds targeting the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by an anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells inhibited adhesion on the extracellular matrix and impaired metastasis in animals (14, 16, 22). These observations suggested that some functions of the IGF-IR must be critical for metastatic cell spread. Here we addressed the possibility that in ER-negative metastatic breast cancer cells, the IGF-IR selectively promotes growth-unrelated processes, such as migration and invasion, but is not engaged in the transmission of growth and survival signals. Using ER-negative MDA-MB-231 breast cancer cells, we set about to delineate IGF-I-dependent pathways involved in migration, and to pinpoint the defects in IGF mitogenic signal. For comparison, the relevant IGF-I responses were analyzed in ER-positive MCF-7 cells.

MATERIALS AND METHODS

Plasmids. The pcDNA3-IGF-IR expression plasmid encoding the wild-type IGF-IR under the CMV promoter was described before (13). The expression plasmids encoding constitutively active forms of Akt kinase, myristylated Akt (MyrAkt) and Akt with an activating point mutation (AktE40K), were obtained from Drs. P. Tsichlis and T. Chan (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) and described were before (23). The Akt plasmids contain the HA-tag allowing for easy identification of Akt transfected cells.

Cell Lines. MDA-MB-231 cells were obtained from ATCC. MDA-MB-231/IGF-IR clones were generated by stable transfection of MDA-MB-231 cells with the plasmid pcDNA3-IGF-IR using a standard calcium phosphate precipitate procedure (13). Transfectants resistant to 1 mg/ml G418 were screened for IGF-IR expression by FACS (Fluorescence-assisted cell sorting) analysis using anti-IGF-IR mouse monoclonal antibody (mAb) alpha-IR3 10 ug/ml (Calbiochem) and fluorescein goat anti-mouse IgG 2 ug/ml (Calbiochem). Cells stained with the secondary antibody alone were used as a control. IGF-IR expression in transfectants was always analyzed in parallel with that in the parental MDA-MB-231 cells and in IGF-IR overexpressing cells MCF-7/IGF-IR clones 12 and 15 (13). IGF-IR levels in MDA-MB-213-derived clones and control cell lines were then confirmed by Western blotting (WB) with specific antibodies (listed below) and by binding assay with (^{125}-I-IGF-I) (as described before in Ref. 13). MCF-7 cells and MCF-7/IGF-IR clone 12 overexpressing the IGF-IR were described in detail previously (12).

Transient Transfection. 70% confluent cultures of MDA-MB-231 and MCF-7 cells were transiently transfected with Akt kinase expression plasmids using Fugene 6 (Roche). Transfection was carried out for 6h; the optimal plasmid/Fugene 6 ratio was 1 ug/3 ul. Upon transfection, the cells were shifted to PRF-SFM and the expression of total and active Akt kinase at 0 (media shift), 2, and 4 days post transfection was assessed by WB with specific antibodies (see below). In parallel, the efficiency of transfection and plasmid expression was monitored by measuring the cellular levels of HA-tag by WB (see below).

Cell Culture. MDA-MB-231 and MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MDA-MB-231- and MCF-7-derived clones overexpressing the IGF-IR were maintained in DMEM:F12 plus 5% CS plus 200 ug/ml G418. In the experiments requiring E2- and serum-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 uM FeSO4 and 2 mM L-glutamine (referred to as PRF-SFM) (13).

Growth Curves. To analyze the growth in serum-containing medium, the cells were plated in 6-well plates in DMEM:F12 (1:1) containing 5% CS at a concentration of 1.5-2.0x10^5 cells/plate; the number of cells was then assessed by direct counting at 1, 2, and 4 days after plating. To study IGF-I-dependent proliferation, the cells were plated in 6-well plates in the growth medium as above. The following day (day 0), the cells at approximately 50% confluence were shifted to PRF-SFM containing 20 ng/ml IGF-I. Cell number was determined at days 1, 2, and 4.

Apoptosis. The cells grown on coverslips in normal growth medium were shifted to PRF-SFM at 70% confluence and then cultured in the presence or absence of 20 ng/ml IGF-I for 0, 1, 2, and 4 days. Apoptosis in the cultures was determined with the In Situ Cell Death Detection kit, Fluorescein (Roche), following manufacturer instructions. The cells containing DNA strand breaks were stained with fluorescein-dUTP, and detected by fluorescence microscopy. Cells that detached during the experiment were spun on glass slides using cytospin and processed as above. Apoptotic index (% number of apoptotic cells/total cell number in a sample field) was determined for adherent and floating cell populations and the indices combined.

Immunoprecipitation and Western Blotting. 70% cultures were shifted to PRF-SFM for 24 h and then stimulated with 20 ng/ml IGF-I for 15 min, 1 day or 2 days. Proteins were obtained by lysing the cells with a buffer containing: 50 mM HEPES pH 7.5, 150 mM, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10% glycerin, 0.2 mM Na_3VO_4, 1% PMSF, 1% aprotinin. The IGF-IR was immunoprecipitated (IP) from 500 ug of protein lysate with anti-IGF-IR mAb (Calbiochem), and subsequently detected by WB with anti-IGF-IR polyclonal Ab (pAb) (Santa Cruz). IRS-1 was precipitated from 500 ug of lysate with anti-IRS-1 pAb (UBI) and detected by WB using the same Ab. Tyrosine phosphorylation (PY) of immunoprecipitated IRS-1 or IGF-IR was assessed by WB with anti-phosphotyrosine mAb PY20 (Transduction Laboratories). IRS-1-associated p85 subunit of PI-3K was detected in IRS-1 immunoprecipitates by WB with anti-p85 mAb (Transduction Laboratories).

Akt, ERK1/ERK2 and p38 MAP kinases (active and total), and active GSK3 were assessed by WB in 50 ug of whole cell lysates with appropriate Abs from New England Biolabs. The expression of HA-tag was probed by WB in 50 ug of protein lysate with anti-HA mAb (Babco). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

IRS-1 Associated PI-3K Activity. PI-3K activity was determined in vitro, as described by us before (24). Briefly, 70 % cultures were synchronized in PRF-SFM for 24h and then stimulated with 20 ng IGF-I for 15 min or 2 days. Untreated cells were used as controls. IRS-1 was precipitated from 500 ug of cell lysates; IRS-1 IPs were then incubated in the presence of inositol and ^{32}P-ATP for 30 min at room temperature. The products of the kinase reaction were analyzed by thin-layer chromatography using TLC plates (Eastman Kodak). Radioactive spots representing PI-3P were visualized by autoradiography, quantified by laser densitometry (ULTRO Scan XL, Pharmacia), and than excised from the plates and counted in a beta-counter.

Motility Assay. Motility was tested in modified Boyden chambers containing porous (8 mm) polycarbonate membranes, as described by us before (10, 25). Briefly, $2x10^4$ cells synchronized in PRF-SFM for 24h were suspended in 200 ul of PRF-SFM and plated into upper chambers. Lower chambers contained 500 ul of PRF-SFM with IGF-I (20 ng/ml). After 24h, the cells in the upper chamber were removed, while the cells that migrated to the lower chamber were fixed and stained in Coomassie Blue solution (0.25g Coomassie blue/45 ml water/45ml methanol/10 ml glacial acetic acid) for 5 min. After that,

the chambers were washed 3 times with H_2O. The cells that migrated to the lower chamber were counted under the microscope.

Inhibitors of PI-3K and MAPK. LY294002 (Biomol Res. Labs) was used to specifically inhibit PI-3K (26). UO126 (Calbiochem) was used to block MEK 1/MEK 2 kinases, and subsequently inhibit ERK1 and ERK2 (27), and SB203580 (Calbiochem) was employed to downregulate p38 MAP kinase (28). To determine the optimal non-toxic concentrations of the compounds, different doses (1-100 uM) of the inhibitors were studied. Additionally, the efficacy of LY294002 and UO126 in inhibiting the phosphorylation of the relevant downstream substrates (Akt and ERK1/ ERK2 kinases, respectively) was determined by WB. This test was not used for SB203580, as its inhibitory action in intact cells is not associated with decreased tyrosine phosphorylation of p38 kinase (28). Ultimately, for both growth and migration experiments LY294002 was used at 50 uM, UO126 at 5 uM, and SB203580 at 10uM.

RESULTS

MDA-MB-231/IGF-IR cells. To study growth related and -unrelated effects of IGF-I in metastatic cells, we used an ER-negative, metastatic breast cancer cells MDA-MB-231. These cells express low levels of the IGF-IR and do not respond to IGF-I with growth (18, 21). Since it has been established that mitogenic response to IGF-I requires a threshold level of IGF-IR expression (e.g., in NIH 3T3-like fibroblasts, ~$1.5x10^4$ IGF-IRs) (29, 30), our first goal was to test whether increasing the cellular content of the IGF-IR would induce IGF-I-dependent growth in MDA-MB-231 cells. To this end, several MDA-

MB-231 clones overexpressing the IGF-IR (MDA-MB-231/IGF-IR cells) were generated by stable transfection, and the receptor content was analyzed by binding assay, FACS analysis (data not shown) and WB (Fig. 1). We determined that MDA-MB-231 clones 2, 21, and 31 express approximately $3x10^4$, $1.5x10^4$, and $2.5x10^5$ IGF-IRs/cell, respectively, while the parental MDA-MB-231 cells express approximately $7x10^3$ IGF-IRs/cell (18). For comparison, ~ $6x10^4$ IGF-IRs were found in ER-positive MCF-7 cells (Fig. 1) (13).

IGF-IR overexpression does not enhance the growth of MDA-MB-231/IGF-IR cells in serum-containing medium. Analysis of growth curves of different MDA-MB-231/IGF-IR clones indicated that overexpression of the IGF-IR never improved proliferation in normal growth medium, and in the case of clone 31 characterized by the highest IGF-IR content, an evident growth retardation at days 2 and 4 ($p<0.05$) was observed (Fig. 2A). By contrast, overexpression of the IGF-IR augmented proliferation of ER-positive MCF-7 cells (Fig. 2B).

IGF-IR overexpression does not promote IGF-I-dependent growth or survival of MDA-MB-321 cells. Subsequent studies established that increasing the levels of the IGF-IR from $7x10^3$ up to $2.5x10^5$ was not sufficient to induce IGF-I-dependent growth response in MDA-MB-231 cells. In fact, similar to the parental cells, all MDA-MB-231/IGF-IR clones were progressively dying in PRF-SFM supplemented with 20 ng/ml (Fig. 3A). Notably, in the clone 31 expressing $2.5x10^5$ IGF-IRs/cells, cell death rate in PRF-SFM with or without IGF-I exceeded that in the parental cells and in other clones with lower IGF-IR levels (Fig. 3A and data not shown). Conversely, in ER-positive cells, the IGF-IR was effectively transducing growth signals, and increasing receptor levels from

$6x10^4$ (MCF-7 cells) to $5x10^5$ (MCF-7/IGF-IR clone 12) significantly ($p<0,05$, day 4) promoted IGF-I-dependent proliferation (Fig. 3B).

The analysis of the anti-apoptotic effects of IGF-I in the above cell lines cultured for 48 h under PRF-SFM indicated that IGF-I reduced apoptosis, by ~3-fold, in ER-positive cells, but it was totally ineffective in MDA-MB-231 and MDA-MB-231/IGF-IR cells (Tab. 1).

IGF-IR signaling in MDA-MB-231 and MDA-MB-231/IGF-IR cells. Next, we investigated, on a molecular level, the basis underlying the lack of IGF-I growth response in ER-negative cells. IGF-I signaling was studied in MDA-MB-231 cells, MDA-MB-231, clone 31, and in parallel, in the control cell lines MCF-7 and MCF-7/IGF-IR clone 12. The experiments focused on IGF-IR tyrosine kinase activity and several postreceptor signaling pathways that are known to control the growth and survival in ER-positive breast cancer cells (and many other cell types), namely the IRS-1/PI-3K, Akt, and ERK1/ERK2 pathways (1, 17, 24, 31, 33). We also probed other IGF-I effectors that have been shown to contribute to non-mitogenic responses in ER-positive breast cancer cells, such as p38 kinase and SHC (10, 25, 34).

Because both acute and chronic effects may be important for biologic IGF-I response (35), we studied IGF-IR signaling at different times following stimulation: 15 min, 1h, 2 days and 4 days. In both ER-positive and -negative cell types, IGF-I signaling seen at 15 min was identical to that at 1h, while IGF-I response at 2 days was similar to that at 4 days. Thus, Fig. 4 demonstrates the representative results obtained with cells stimulated for 15 min and 2 days.

In MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-IR and its major substrate, IRS-1, were tyrosine phosphorylated at both time-points in a manner roughly reflecting the receptor levels. The activation of both molecules was stronger just after stimulation and weaker at 2 days of treatment (Fig. 4A). Analogous IGF-I effects were seen in MCF-7 cells and their IGF-IR-overexpressing derivatives (Fig. 4B).

One of the major growth/survival pathways initiated at IRS-1 is the PI-3K pathway (31, 36). In all cell lines studied, the p85 regulatory subunit of PI-3K was found associated with IRS-1 at 15 min and 2 days (Fig. 4A and B), which suggested a continuos stimulation of PI-3K. However, subsequent measurements of IRS-1-associated PI-3K activity in vitro demonstrated that p85/IRS-1 binding at later time points is not a direct marker of enzyme stimulation. Specifically, at 15 min after IGF-I addition, PI-3K activity was similar in both cell types, but at 2 days, in MDA-MB-231 and MDA-MB-231/IGF-IR cells, IGF-I did not stimulate PI-3K through IRS-1, or induced it very weakly, while in MCF-7 and MCF-7/IGF-IR cells, a good level of PI-3K activation was observed (Fig. 5).

The in vitro activity of PI-3K was reflected by the stimulation of its downstream effector Akt kinase. At 15 min, Akt was upregulated in response to IGF-I an all cell lines, but at 2 days, no effects of IGF-I were seen in MDA-MB-231 and MDA-MB-231/IGF-IR cells, while upregulation of Akt was still evident in MCF-7 and MCF-7/IGF-IR cells (Fig. 4C and D). Akt is known to phosphorylate (on Ser9) and downregulate the glycogen synthase kinase GSK3-beta (23, 31, 33). We found that in both cell types, the phosphorylation of GSK-3 beta reflected the dynamics of Akt activity, with no induction of phosphorylation observed at 2 days in ER-negative cells (Fig. 4C) and IGF-I-stimulated

phosphorylation in MCF-7 and MCF-7/IGF-IR cells (by 40 and 120%, respectively) (Fig. 4D).

Another effector pathway of IGF-I that is important in growth and survival involves ERK1 and ERK2 kinases (1, 35, 37). This pathway was strongly upregulated at 15 min and weakly induced at 2 days in MCF-7 and MCF-7/IGF-IR cells. In MDA-MB-231 and MDA-MB-231/IGF-IR cells, the basal activation of ERK1/2 kinases was always high, and the addition of IGF-I only minimally (10-20%) induced the enzymes at 15 min, with no effects seen at 2 days (Fig. 4E and F).

p38, a stress-induced MAP kinase, and a known mediator of non-growth responses in breast cancer cells (34), was strongly stimulated by IGF-I in ER-negative cells at 15 min (Fig. 4E). By contrast, in ER-positive cells, the enzyme was induced only at 2 days (Fig. 4F). The stimulation of SHC, a substrate of the IGF-IR involved in migration and growth in ER-positive cells (10, 25), was weak in all cell types and no differences in the activation patterns were observed (data not shown).

Reactivation of Akt kinase in MDA-MB-231 cells. Previous results indicated that MDA-MB-231 and MDA-MB-231/IGF-IR cells are unable to sustain IGF-I-dependent activation of the PI-3K/Akt survival pathway when cultured in the absence of serum for 2-4 days. Consequently, we tested whether cell death under PRF-SFM conditions can be reversed by forced overexpression Akt kinase. Two different expression plasmids encoding constitutively active forms of Akt kinase, Myr-Akt and Akt/E40K, (23) were transiently transfected into MDA-MB-231 cells, and as a control, into MCF-7 cells. The increased expression of Akt, without any concomitant cytotoxicity, was evident in all

cases (Fig. 6A and data not shown), however; no significant improvement in the growth or survival with or without IGF-I in ER-negative cells was observed (Fig. 6B and data not shown). A tendency of MDA-MB-231 cells to survive better at 2 days post transfection (at the time of the greatest Akt activity) was noted, but the differences did not reach the statistical significance ($p>0.05$). This suggested that although improper Akt stimulation may be associated with the lack of IGF-I mitogenic response in ER-negative cells, some other pathways must also be responsible for growth and survival.

Inhibition of IGF-IR signaling pathways. To complement the above studies, we examined the importance of the PI-3K, ERK1/ERK2, and p38 kinase pathways in IGF-I-dependent growth and survival of ER-positive and ER-negative breast cancer cells using specific inhibitors (26-28). The efficacy of PI-3K and ERK1/ERK2 inhibitors was first tested by establishing their effects on the activity of target proteins (Fig. 7). Tab. 2 demonstrates that the inhibition of PI-3K with LY294002 reduced the growth of MCF-7 and MCF-7/IGF-IR cells, but did not have significant impact or had only minimal effects on MDA-MB-231 and MDA-MB-231/IGF-IR cells. Furthermore, the action of LY294002 was counteracted by IGF-I in ER-positive, but not in ER-negative cells.

The inhibition of ERK1/ERK2 with UO126, a compound targeting the upstream kinases MEK1/MEK2, affected the growth and/or survival in both cell types, but only in MCF-7 and MCF-7/IGF-IR cells, IGF-I was able to oppose this effect. Targeting p38 kinase with SB203580 reduced survival of MDA-MB-231 and MDA-MB-231/IGF-IR cells, and to a lesser extent the growth and survival of MCF-7 and MCF-7/IGF-IR cells.

IGF-I was not able to reverse the anti-mitogenic action of the p38 kinase inhibitor in either of the cell lines studied (Tab. 2).

Cumulatively, these results suggested that in ER-positive cells, IGF-I transmits mitogenic signals through PI-3K and ERK1/ERK2 pathways. By contrast, IGF-I does not induce growth or survival signal through these pathways in ER-negative cells.

IGF-I stimulates migration in MDA-MB-231 cells. Next, we investigated the non-mitogenic effects of IGF-I in ER-negative and ER-positive breast cancer cells. Unlike with the growth and survival responses, we found that IGF-IR was an effective transducer of non-mitogenic signals in MDA-MB-231 and MDA-MB-231/IGF-IR cells. Specifically, in the chemoattraction experiments, IGF-I placed in the lower chamber was stimulating migration of ER-negative cells in a manner reflecting IGF-IR content. In parallel experiments, the same doses of IGF-I induced migration in ER-positive cells (Tab. 3). The addition of IGF-I to the upper chamber always suppressed migration of all cell lines (data not shown).

IGF-I pathways regulating migration of MDA-MB-231 cells. Using the inhibitors of PI-3K, ERK1/ERK2, and p38 kinases, we determined which pathways of the IGF-IR are involved in migration of ER-positive and ER-negative cells. As demonstrated in Tab. 4, downregulation of PI-3K with LY294002 inhibited basal migration of both cell types, with a more pronounced effect in ER-negative cells. Similarly, blockade of p38 kinase reduced motility of all cell lines studied. Interestingly, inhibition of ERK1 and ERK2 with UO126 resulted in the stimulation of migration in both ER-positive and ER-negative cells. The addition of IGF-I as a chemoattractant significantly counteracted the effects of all

3 inhibitors, however, no clear association between the cellular levels of the IGF-IR and this competing action of IGF-I was noted (Tab. 4). These results suggested that IGF-I-dependent motility in both types of cells requires the PI-3K and p38 kinase pathways, and may be normally suppressed by the activation of ERK1/ERK2 kinases.

DISCUSSION

The experimental and clinical evidence supports the notion that hypercativation of the IGF-IR may be critical in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages of the disease, including metastasis, is still obscure (1). For instance, whereas the IGF-IR has been found overexpressed in primary breast tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease (1). According to Pezzino et al., who studied the IGF-IR status in two patient subgroups representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population, there is a highly significant correlation between IGF-IR expression and better prognosis (38). Similar conclusions were reached by Peyrat and Bonneterre (39). Therefore, it has been proposed that similar to the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/ IGF-IR-positive tumors (1, 39). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (40).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good IGF-IR expression (1, 18, 39). By contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and generally do not respond to IGF-I with growth (1, 18-21). However, many ER-negative cell lines appear to depend on the IGF-IR for tumorigenesis and metastasis. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal models (1, 14, 16, 22). These observations suggest that some growth-unrelated pathways of the IGF-IR may be operative in the context of ER-negative cells.

Here we studied whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the non-mitogenic function of the IGF-IR, such as cell migration. Our experiments indicated that the IGF-IR is an effective mediator of cell motility. Furthermore, IGF-I-induced migration was proportional to IGF-IR content. We demonstrated, for the first time, that in MDA-MB-231 ER-negative cells, IGF-IR signaling pathways responsible for cell movement include PI-3 and p38 kinases. Indeed, an acute IGF-I stimulation of MDA-MB-231 and MDA-MB-231/IGF-IR cells appears to induce both PI-3K and p38 kinases, suggesting that this short-time activation may be involved in migration. Both of these pathways have been previously shown to regulate cell motility in breast cancer cells and other cell types (34, 41). Interestingly, the migration of both ER-negative and ER-positive cells was stimulated in the presence of a specific MEK1/MEK2 inhibitor UO126. We observed this effect over a broad range of UO126 doses (1-10 uM)

and in several MDA-MB-231- and MCF-7-derived clones; the same doses always reduced IGF-I-dependent phosphorylation of ERK1/ERK1 (Fig. 7) and supressed cell proliferation in serum-containing medium and PRF-SFM (data not shown and Tab. 2). A slight stimulation of migration in MDA-MB-231, but not in MCF-7 cells, was also observed with another MEK (and ERK1/ERK2) inhibitor PD98059 at 5 uM (Surmacz, unpublished data). These peculiar effects suggest that normally the ERK1/ERK2 pathway positively regulates cell growth and survival, and negatively impacts on cell migration.

In contrast with the positive effects of IGF-I on cell motility in ER-negative and ER-positive breast cancer cells, this growth factor never stimulated the proliferation of MDA-MB-231 cells, while it induced the growth of MCF-7 cells and MCF-7-derived clones overexpressing the IGF-IR. It is has been established by Rubini et al. (29) and Reiss et al. (30) that mitogenic response to IGF-I requires a threshold level of IGF-IR expression (in fibroblasts, ~$1.5x10^4$). However, we demonstrated that increasing the levels of IGF-IR from ~$7x10^3$ up to ~$2.5x10^5$ and subsequent upregulation of IGF-IR tyrosine phosphorylation was not sufficient to induce the growth of MDA-MB-213 cells in IGF-I. Similar results were obtained by Jackson and Yee, who showed that overexpression of IRS-1 in ER-negative MDA-MB-435A and MDA-MB-468 breast cancer cells did not stimulate IGF-I-dependent mitogenicity (20). These authors suggested that the lack of IGF-I response, even in IRS-1 overexpressing ER-negative cells, was related to insufficient stimulation of ERK1/ERK2 and PI-3K pathways (20). Defective insulin response in ER-negative cell lines has also been described by Costantino et al. and linked with an increased tyrosine phosphatase activity (42).

Our experiments suggest that the lack of IGF-I mitogenicity in MDA-MB-231 and MDA-MB-231/IGF-IR cells is not related to the impaired IGF-IR or IRS-1 tyrosine phosphorylation. The cells are also able to respond to an acute IGF-I stimulation with a good activation of the PI-3K/Akt and ERK-1/ERK2 pathways. We hypothesize that this transient stimulation could be sufficient to induce some IGF-I response, such as cell motility. Mitogenic response, on the other hand, may rely on a more sustained activation of critical IGF-IR signals, as demonstrated by Swantek and Baserga in mouse embryo fibroblasts (35). Indeed, the most significant difference in IGF-I signal between ER-negative and ER-positive cells rested in the impaired long-term (2 days) stimulation of the PI-3K/Akt pathway: MDA-MB-231 and MDA-MB-231/IGF-IR cells were unable to sustain this IGF-I-induced signal for 2 days, while in MCF-7 and MCF-7/IGF-IR cells, the PI-3K/Akt pathway was still active at this time.

The subsequent experiments, however, demonstrated that increasing Akt activity is not sufficient to stimulate the survival or growth (with or without IGF-I) of ER-negative cells, which suggested that while Akt could be important in these processes, other pathways are also necessary. The identity of these pathways in presently unknown, but we obtained preliminary results indicating that the survival and growth of MDA-MB-231 cells can be significantly improved by the overexpression of an IGF-IR truncated at either aa 1229 or aa 1245 (Morelli and Surmacz, unpublished data). The anti-apoptotic effects induced by truncation of the IGF-IR have been described in other cell systems, but their molecular bases are not known (43).

In summary, our data suggest that IGF-IR signaling and function undergo evolution during breast cancer progression. In ER-positive cells, IGF-IR transmits various signals, such as growth, survival, migration, adhesion. In ER-negative cells, the growth-related functions of the IGF-IR become attenuated, but the receptor is still able to control non-mitogenic processes, such as migration. It is likely that this kind of evolution concerns also the response to other growth factors. Epidermal growth factor, for instance, is an effective mitogen for ER-positive breast cancer cells, but does not stimulate the proliferation or survival in MDA-MB-231 cells, despite high EGF-R expression (44). However, as recently demonstrated by Price et al., EGF is a potent chemoattractant for MDA-MB-231 cells. EGF-induced migration in MDA-MB-231 cells requires PI-3K and PLC gamma and is not inhibited by antagonists of ERK1/ERK2 (44).

In conclusion, mitogenic and non-mitogenic pathways induced by growth factors in breast cancer cells may be dissociated, and attenuation of one is not necessarily linked with the cessation of the other. Delineating the non-mitogenic responses will be critical for the development of drugs specifically targeting metastatic cells.

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TABLES

Tab. 1. Effects of IGF-I on apoptosis in ER-negative and ER-positive cells.

Cell Line	*Apoptosis (%)*	
	SFM	*SFM+IGF-I*
MDA-MB-231	**41.4**±3.0	**46.0**±1.9
MDA-MB-231/IGF-IR	**50.1**±4.1	**53.3**±4.2
MCF-7	**14.5**±0.2	**4.2**±0.1
MCF-7/IGF-IR	**10.1**±1.3	**2.8**±0.1

Apoptosis was studied in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells and MCF-7/IGF-IR clone 12. The cells were cultured for 48 h in PRF-SFM, and the apoptotic index (% apoptotic cells/total cell number in the field) was determined by TUNEL, as described under Materials and Methods. The results are average from at least 3 experiments; SD values are given.

Tab. 2. Effects of PI-3K and MAPK inhibitors on growth and survival of ER-negative and ER-positive breast cancer cells.

Cell Line	*Inhibition (%)*					
	LY294002 (PI-3K)		*UO126 (MEK)*		*SB203580 (p38)*	
	SFM	**+IGF**	**SFM**	**+IGF**	**SFM**	**+IGF**
MDA-MB-231	**9.4**±1.0	**7.8**±0.8	**35.0**±2.6	**39.0**±2.7	**47.8**±2.2	**42.5**±4.4
MDA-MB-231/IGF-IR	**11.1** ±1.2	**12.3**±0.9	**18.3**±0.9	**22.9**±1.3	**29.5**±2.0	**35.6**±3.6
MCF-7	**68.8**±3.3	**35.0**±1.2	**42.6**±3.8	**26.3**±2.5	**11.7**±1.2	**10.0**±0.4
MCF-7/IGF-IR	**73.2**±6.7	**34.6**±2.7	**49.4**±3.9	**20.2**±1.5	**24.7**±0.2	**25.9**±0.9

MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were cultured in PRF-SFM with or without IGF-I in the presence or absence of the inhibitors for 2 days, as described under Materials and Methods. The difference (in percentages) between the number of live cells under treatment and the number of cells cultured under control conditions was defined as inhibition (%). The results are average from 3 experiments; SDs are given.

Tab. 3. IGF-I-induced migration in ER-negative and ER-positive breast cancer cells.

Cell Line	*IGF-I-Induced Migration (%)*
MDA-MB-231	29±3.0
MDA-MB-231/IGF-IR	74±4.5
MCF-7	11±0.2
MCF-7/IGF-IR	30±2.9

The migration of MDA-MB-231 and MCF-7 cells as well their IGF-IR overexpressing derivatives, MDA-MB-231/IGF-IR clone 31 and MCF-7/IGF-IR clone 12 was determined as described under Materials and Methods. The results are average (±SD) from at least 5 experiments.

Tab. 4. Effects of PI-3K and MAPK inhibitors on migration of ER-negative and positive breast cancer cells.

Cell Line	*Change (%)*					
	LY294002 (PI-3K)		*UO126 (MEK)*		*SB203580 (p38)*	
	SFM	**+IGF**	**SFM**	**+IGF**	**SFM**	**+IGF**
MDA-MB-231	**-47.2**±3.3	**-13.3**±1.0	**+53.4**±3.5	**+36.4**±2.2	**-30.2**±2.9	**-8.5**±0.7
MDA-MB-231/IGF-IR	**-41.0**±4.2	**-9.2**±0.4	**+29.0**±2.0	**+12.6**±0.7	**-40.1**±0.4	**-2.5**±0.0
MCF-7	**-15.4**±0.8	**-8.7**±0.2	**+94.9**±3.9	**+56.4**±1.7	**-18.9**±1.1	**-5.6**±0.2
MCF-7/IGF-IR	**-33.1**±2.7	**-12.8**±0.3	**+65.6**±5.4	**+23.8**±1.9	**-24.8**±0.8	**-1.7**±0.1

The migration of MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 was tested in modified Boyden chambers as described under Materials and Methods. The inhibitors were added to the cells in the upper chamber at the time of cell plating, and their effect on basal (SFM) or IGF-I-induced (+IGF) migration was assessed 24h later. The values represent % change relative to the migration at basal conditions in PRF-SFM (SFM) without inhibitors or chemoattractants. The experiments were repeated at least 3 times; the average results ±SD are given.

LEGENDS TO FIGURES

Fig. 1. MDA-MB-231/IGF-IR clones.

MDA-MB-231/IGF-IR cells were generated by stable transfection with an IGF-IR expression vector, as described under Materials and Methods. In several G418-resistant clones, the expression of the IGF-IR protein was tested by WB with anti-beta subunit IGF-IR pAb (Santa Cruz) in 50 ug of total protein lysate. For comparison, MCF-7 cells and MCF-7/IGF-IR clone 15 with known levels of IGF-IR ($6x10^4$ and $3x10^6$, respectively) (13) are shown. Low levels of IGF-IR in MDA-MD-231 cells (~$7x10^3$ receptors/cell) are not visible in this blot, but were detectable in its phosphorylated form by IP and WB in 500 ug of protein lysates (see Fig. 4A). The estimated expression of the IGF-IR in clones 2, 21, and 31 is $1.5x10^4$, $3x10^4$, and $2.5x10^5$ receptors/cell, respectively.

Fig. 2. Effect of IGF-IR overexpression on the growth of ER-negative and ER-positive cells in serum-containing medium.

MDA-MB-231 cells, MDA-MB-231/IGF-IR clones 2, 21, and 31 **(A)**, and their ER-positive counterparts, MCF-7 cells and MCF-7/IGF-IR, clones 12 and 15 **(B)**, were plated in DMEM:F12 plus 5% CS. The cells were counted at 50% confluence (day 0), and at subsequent days 1, 2, and 4. The results are average from 3 experiments.

Fig. 3. IGF-I-dependent growth and survival of ER-negative and ER-positive breast cancer cells.

MDA-MB-231 cells and MDA-MB-231/IGF-IR clone 31 **(A)** as well as MCF-7 cells and MCF-7/IGF-IR clone 12 **(B)** were synchronized in PRF-SFM and treated with IGF-I, as described in Materials and Methods. The cells were counted at days 0, 1, 2, and 4 of treatment. The results are average from at least 3 experiments.

Fig. 4. IGF-I signaling in ER-negative and ER-positive breast cancer cells.

The activation of IGF-IR/IRS-1/PI-3K signaling **(A** and **B)**, Akt/GSK-3 signaling **(C and D),** and ERK1/ERK2 and p38 kinase signaling **(E** and **F)** was tested in MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12. The cells were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. The cellular levels of the IGF-IR and IRS-1 were detected by IP and WB in 500 ug of total protein lysate using specific antibodies (Materials and Methods). IGF-IR and IRS-1 tyrosine phosphorylation (PY) was assessed upon striping and reprobing the same filters with the anti-PY20 antibody. The levels of IRS-1-bound p85 subunit of PI-3K (p85/IRS-1) were analyzed in IRS-1 IPs by WB with anti-p85 Ab. The levels and activity of Akt, GSK-3, ERK1/ERK2, and p38 kinases were probed by WB in 50 ug of total cellular lysates using specific Abs. The Abs used are listed under Materials and Methods. Representative results are shown. Note decreased IRS-1 expression under 15 min IGF-I treatment in ER-positive cells, as described before (46).

Fig. 5. IGF-I-induced PI-3 kinase activity in ER-negative and ER-positive cells.

MDA-MB-231 cells, MDA-MB-231/IGF-IR clone 31, MCF-7 cells, and MCF-7/IGF-IR clone 12 were synchronized in PRF-SFM and treated with IGF-I for 15 min or 2 days. IRS-1-bound PI-3K activity was measured in vitro in IRS-1 IPs as described under Materials and Methods. The experiments were repeated at least 3 times; the bars indicate SDs.

Fig. 6. Effect of increased Akt activity on the survival of MDA-MB-231 cells.

MDA-MB-231 cells were transiently transfected with expression plasmids encoding 2 different constitutively active Akt kinase mutants (Myr-Akt and Akt/E40K). The Akt vectors contained HA-tag for easy detection. The cells treated with the transfection mixture lacking plasmid DNA (Mock) served as control. The expression of the plasmids as well as the activity and the levels of Akt kinase in the transfected cells were monitored at 2 and 4 days after transfection. 50 ug of total protein lysates were sequentially probed by WB with anti-HA, anti-active Akt, and then anti-total Akt specific Abs (described under Materials and Methods). Representative results are shown **(A)**.

In parallel, the number of cells was assessed at days 0 (post-transfection media change), 1, 2, and 4 after transfection. The results are average from at least 3 times. For the clarity of the graph, the SDs are not pictured **(B)**.

Fig. 7. PI-3K and ERK1/ERK2 kinase inhibitors.

Synchronized cultures of MDA-MB-231 and MCF-7 cells were treated with LY294002 or UO126 in the presence or absence of IGF-I for 15 min, as described under Materials and Methods. The activities of Akt kinase, a downstream substrate of PI-3K, and ERK1/ERK2 kinases were determined by WB in 50 ug of protein lysates using specific antibodies. Representative results are shown.

ABBREVIATIONS

CS, calf serum; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FACS, fluorescence-assisted cell sorting; GSK-3, glycogen synthase kinase-3; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IP, immunoprecipitation; IRS-1, insulin receptor substrate 1; mAb, monoclonal antibody; MAPK, mitogen activated kinases; pAb, polyclonal antibody; PI-3K, phosphatidyl inositol 3-kinase; PLC-gamma, phospholipase C gamma; PgR, progesteron receptor; PRF-SFM, phenol red-free serum-free medium; PY, tyrosine phosphorylation; SFM, serum free medium; WB, Western blotting.

FIGURE 1

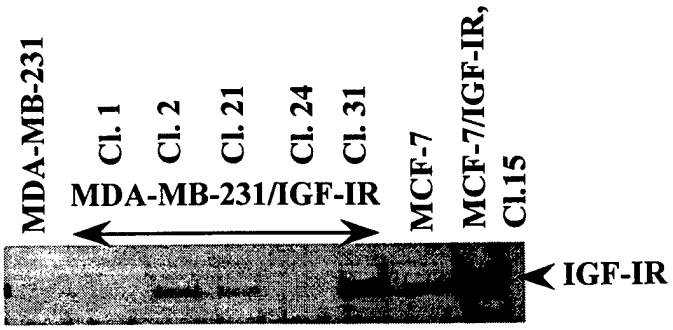

MDA-MB-231
Cl. 1
Cl. 2
Cl. 21
Cl. 24
Cl. 31
MDA-MB-231/IGF-IR
MCF-7
MCF-7/IGF-IR, Cl.15
IGF-IR

FIGURE 2

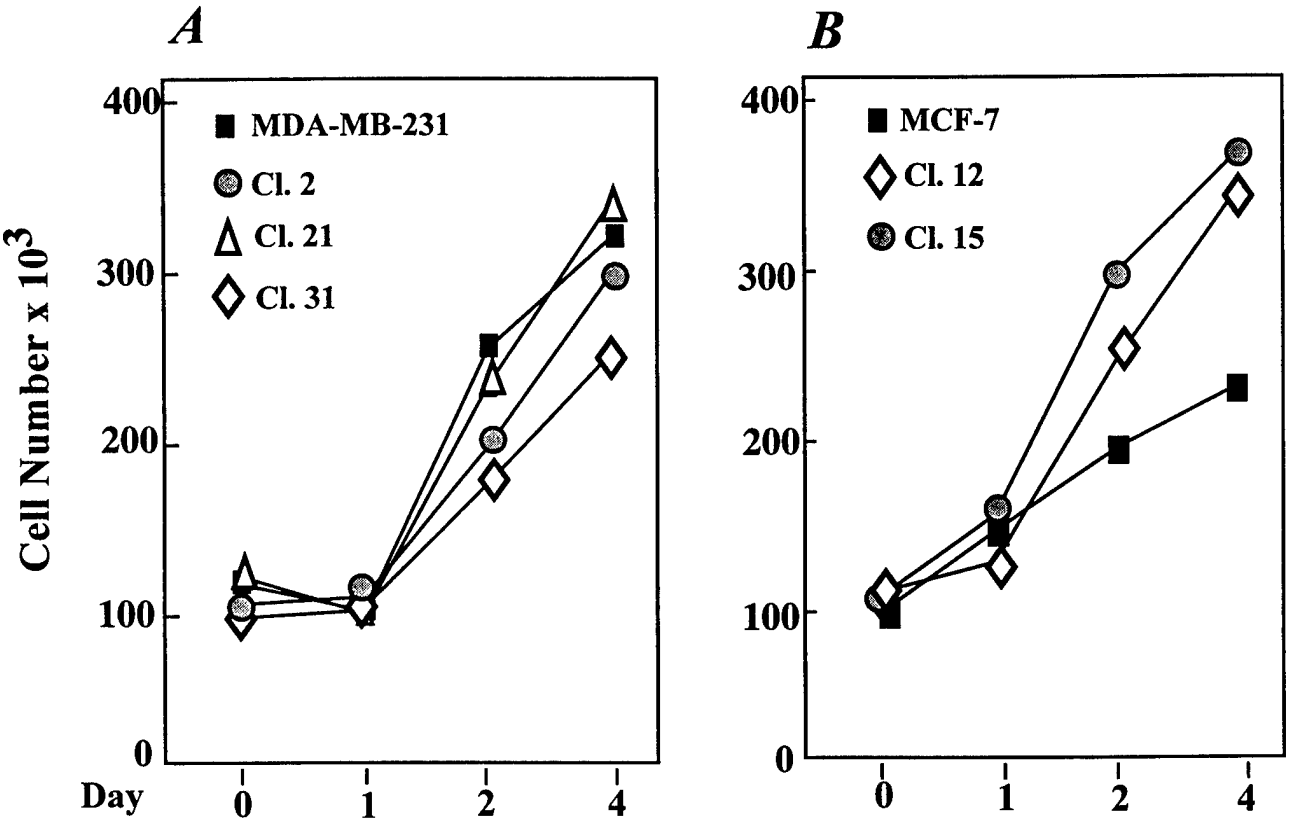

A
B
400
300
200
100
0
Cell Number x 10^3
MDA-MB-231
Cl. 2
Cl. 21
Cl. 31
MCF-7
Cl. 12
Cl. 15
Day
0
1
2
4

FIGURE 3

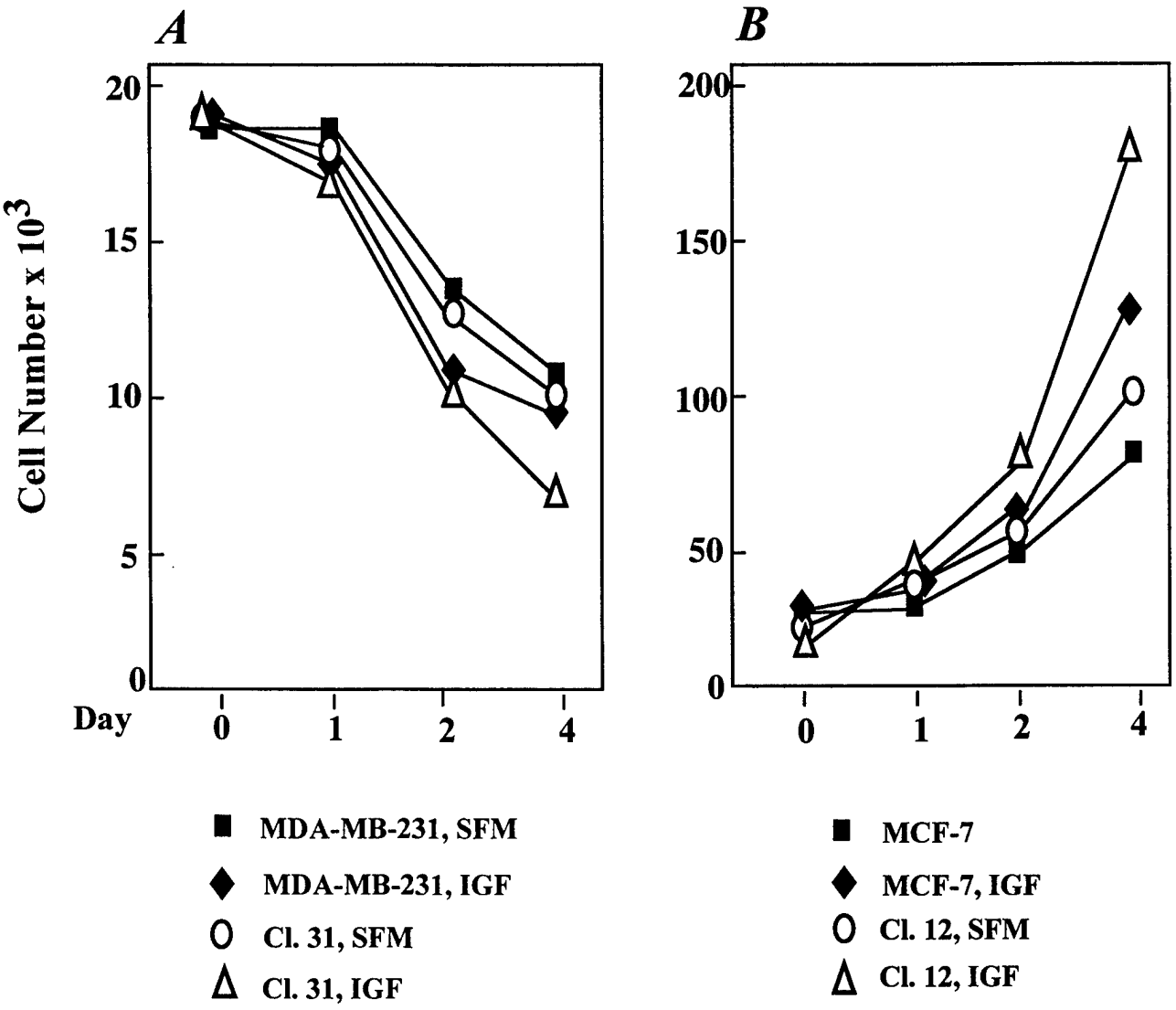

A
B
Cell Number x 10^3
20
15
10
5
0
Day
0
1
2
4
200
150
100
50
0
0
1
2
4
MDA-MB-231, SFM
MDA-MB-231, IGF
Cl. 31, SFM
Cl. 31, IGF
MCF-7
MCF-7, IGF
Cl. 12, SFM
Cl. 12, IGF

FIGURE 4

A

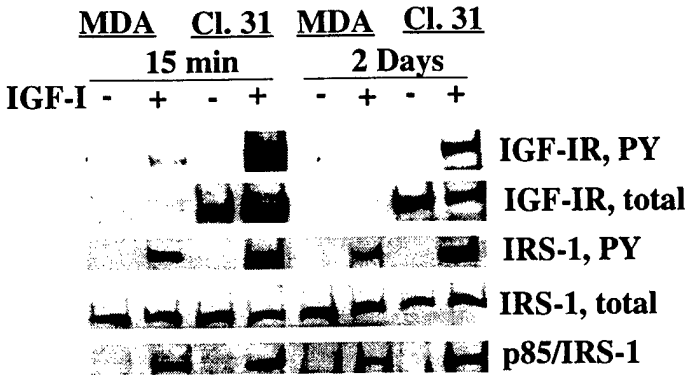

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
IGF-IR, PY
IGF-IR, total
IRS-1, PY
IRS-1, total
p85/IRS-1

FIGURE 4

B

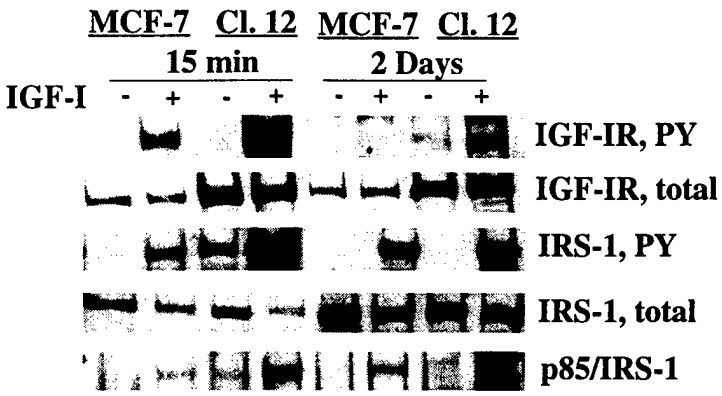

MCF-7
Cl. 12
MCF-7
Cl. 12
15 min
2 Days
IGF-I
- + - + - + - +
IGF-IR, PY
IGF-IR, total
IRS-1, PY
IRS-1, total
p85/IRS-1

FIGURE 4

C

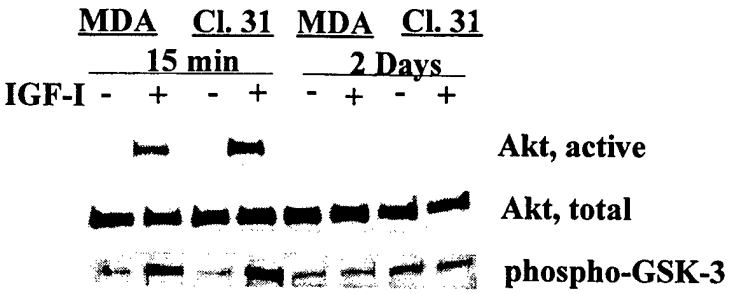

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
Akt, active
Akt, total
phospho-GSK-3

FIGURE 4

D

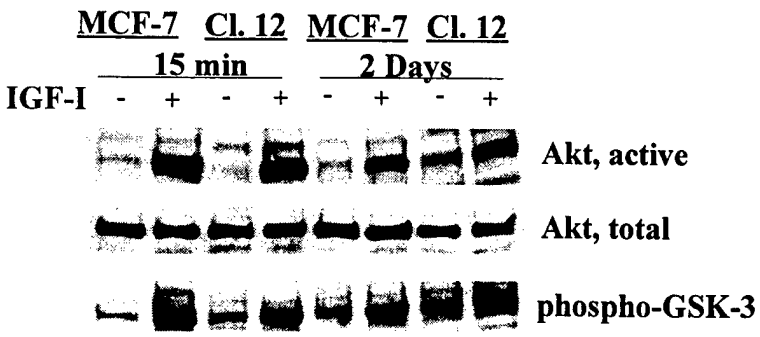

MCF-7
Cl. 12
MCF-7
Cl. 12
15 min
2 Days
IGF-I - + - + - + - +
Akt, active
Akt, total
phospho-GSK-3

FIGURE 4

E

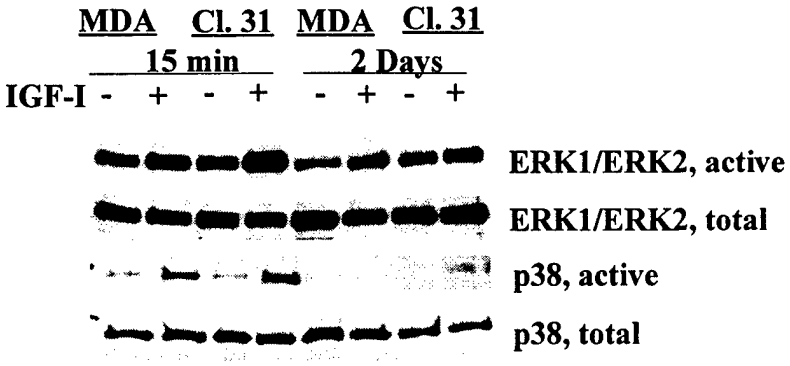

MDA
Cl. 31
MDA
Cl. 31
15 min
2 Days
IGF-I - + - + - + - +
ERK1/ERK2, active
ERK1/ERK2, total
p38, active
p38, total

FIGURE 4

F

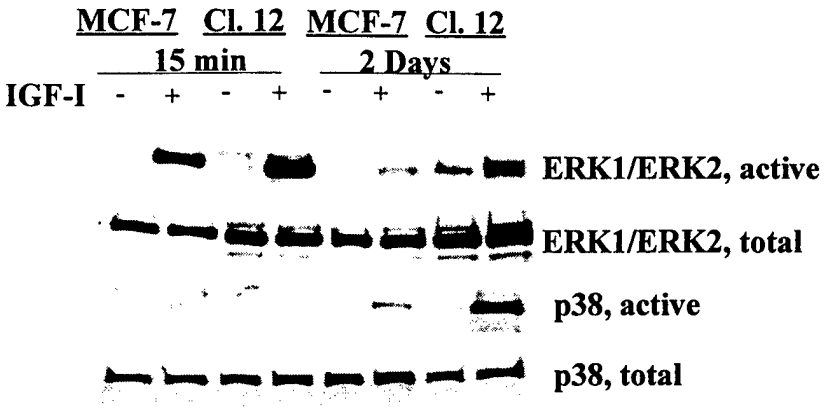
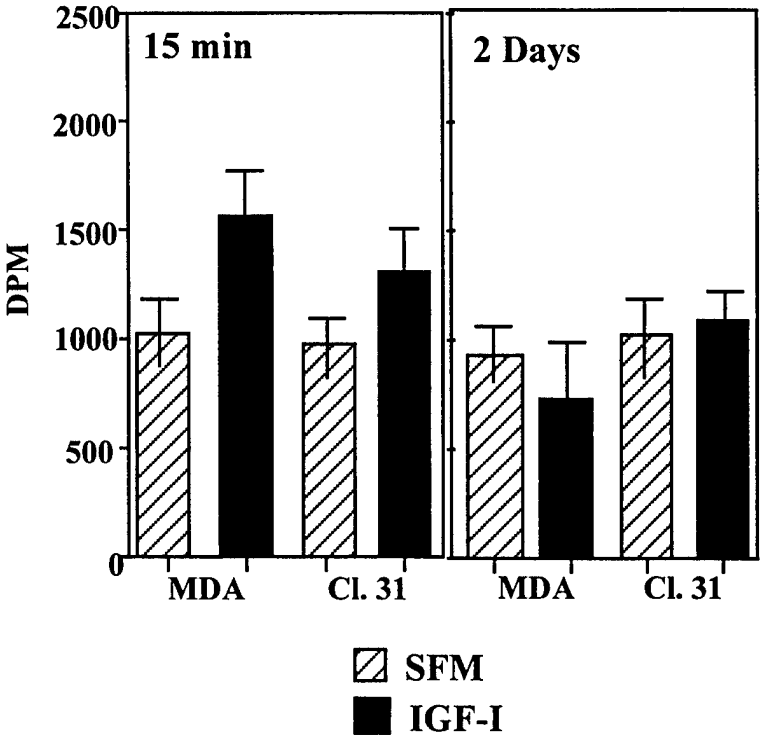

MCF-7
Cl. 12
MCF-7
Cl. 12
15 min
2 Days
IGF-I - + - + - + - +
ERK1/ERK2, active
ERK1/ERK2, total
p38, active
p38, total

FIGURE 5


2500
2000
1500
1000
500
0
DPM
15 min
2 Days
MDA
Cl. 31
MDA
Cl. 31
SFM
IGF-I

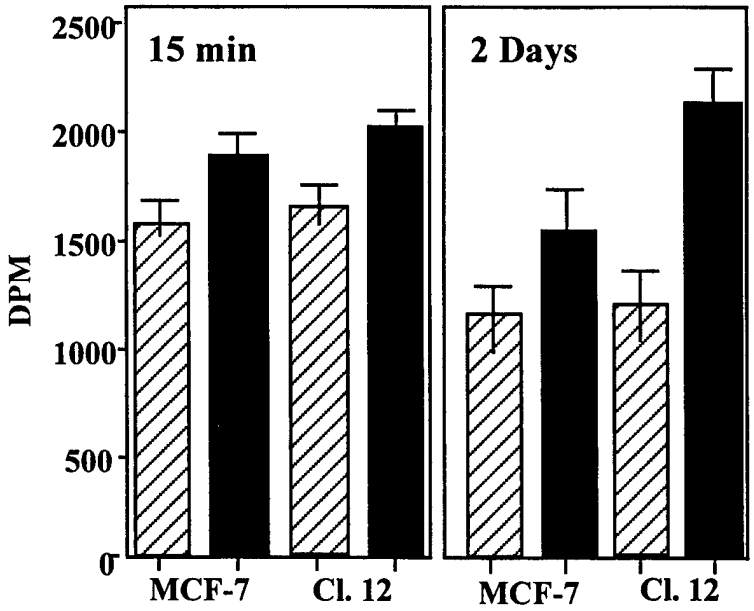

2500
2000
1500
1000
500
0
DPM
15 min
2 Days
MCF-7
Cl. 12
MCF-7
Cl. 12

FIGURE 6

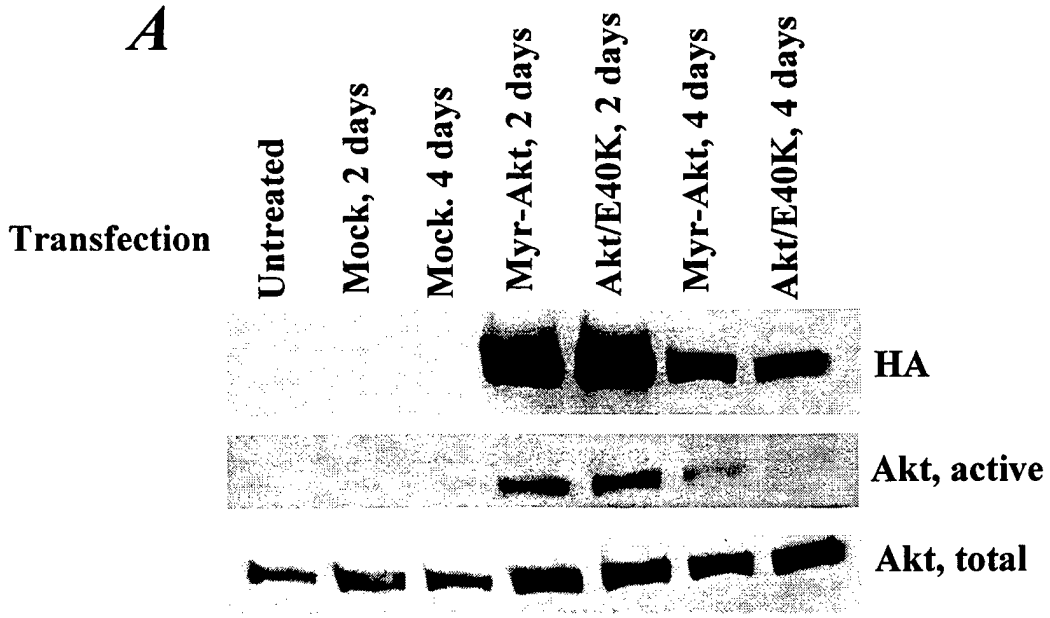

A
Transfection
Untreated
Mock, 2 days
Mock. 4 days
Myr-Akt, 2 days
Akt/E40K, 2 days
Myr-Akt, 4 days
Akt/E40K, 4 days
HA
Akt, active
Akt, total

FIGURE 6

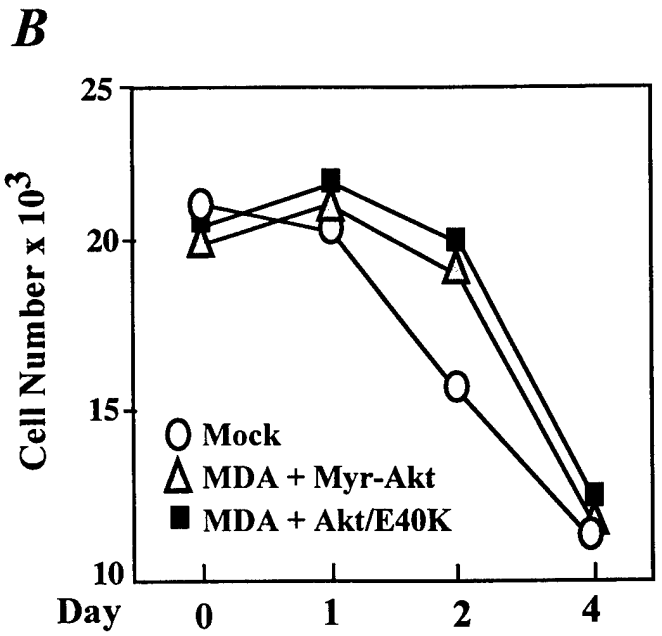
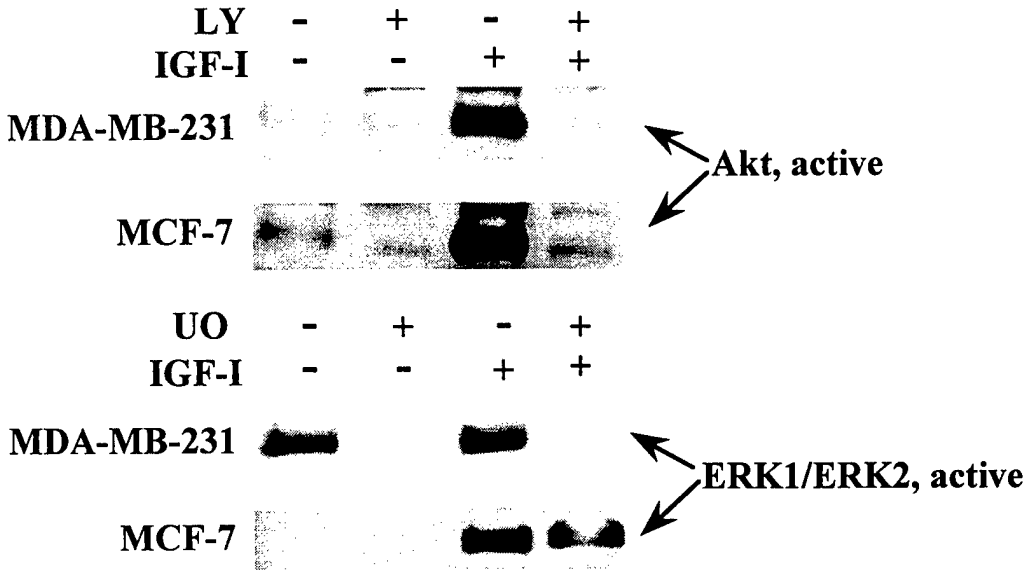

B
25
20
15
10
Cell Number x 10^3
Mock
MDA + Myr-Akt
MDA + Akt/E40K
Day
0
1
2
4

FIGURE 7


LY - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
Akt, active
UO - + - +
IGF-I - - + +
MDA-MB-231
MCF-7
ERK1/ERK2, active

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Function of the IGF-I Receptor in Breast Cancer

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The insulin-like growth factor-I receptor (IGF-IR)[3] is a transmembrane tyrosine kinase regulating various biological processes such as proliferation, survival, transformation, differentiation, cell-cell and cell-substrate interactions. Different signaling pathways may underlie these pleiotropic effects. The specific pathways engaged depend on the number of activated IGF-IRs, availability of intracellular signal transducers, the action of negative regulators, and is influenced by extracellular modulators. Experimental and clinical data implicate the IGF-IR in breast cancer etiology. There is strong evidence linking hyperactivation of the IGF-IR with the early stages of breast cancer. In primary breast tumors, the IGF-IR is overexpressed and hyperphosphorylated, which correlates with radio-resistance and tumor recurrence. *In vitro,* the IGF-IR is often required for mitogenesis and transformation, and its overexpression or activation counteract effects of various pro-apoptotic treatments. In hormone-responsive breast cancer cells, IGF-IR function is strongly linked with estrogen receptor (ER) action. The IGF-IR and the ER are co-expressed in breast tumors. Moreover, estrogens stimulate the expression of the IGF-IR and its major signaling substrate IRS-1, while antiestrogens downregulate IGF-IR signaling, mainly by decreasing IRS-1 expression and function. On the other hand, overexpression of IRS-1 promotes estrogen-independence for growth and transformation. In ER-negative breast cancer cells, usually displaying a more aggressive phenotype, the levels of the IGF-IR and IRS-1 are often low and IGF is not mitogenic, yet the IGF-IR is still required for metastatic spread. Consequently, IGF-IR function in the late stages of breast cancer remains one of the most important questions to be addressed before rational anti-IGF-IR therapies are developed.

KEY WORDS: Breast cancer; insulin-like growth factor I receptor; IRS-1; estrogen-independence; antiestrogen; metastasis.

INTRODUCTION

The insulin-like growth factors I and II (IGFs) act as endocrine, paracrine or autocrine regulators of various biological processes in normal and neoplastic cells. The actions of IGF-I in the adult are mediated primarily by the type I insulin-like growth factor receptor (IGF-IR), while IGF-II stimulates both the IGF-IR and the insulin receptor (IR) (1,2). It has been well established that in many cell types, activation of the IGF-IR is essential for cell survival, transformation, and hormone-independence—the processes that pro-

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[3] Abbreviations: three-dimensional (3-D); amino acid; (aa); disease-free survival (DFS); E-cadherin (E-cad); 17-beta estradiol (E2); extracellular matrix (ECM); epidermal growth factor receptor (EGFR); estrogen receptor (ER); histidine (His); insulin-like growth factor (IGF); IGF-I receptor (IGF-IR); insulin receptor (IR); insulin receptor substrate (IRS); lysine (Lys); overall survival (OS); phosphatidylinositol-3 kinase (PI-3K); progesterone receptor (PgR); protein kinase C (PKC); phosphotyrosine binding domain (PTB); MCF-7 cells expressing anti-IRS-1 and anti-SHC RNA, respectively (MCF-7/anti-IRS-1 and MCF-7/anti-SHC); MCF-7 cells overexpressing the IGF-IR, IRS-1, and SHC, respectively (MCF-7/IGF-IR, MCF-7/IRS-1, and MCF-7/SHC); serine (Ser); src-homology 2 domain (SH2); src/collagen homology proteins (SHC); Tamoxifen (Tam).

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mote tumorigenesis (3–6). During the past several years, the impact of the IGF-IR on breast cancer development and progression has also been recognized, providing a new direction for the design of anti-growth factor compounds for breast cancer therapy.

IGF-IR EXPRESSION AND STRUCTURE

Almost all cell types, except hepatocytes and T-lymphocytes, express the IGF-IR (1,3). The IGF-IR is encoded by a 100 kb gene containing 21 exons located on the distal arm of chromosome 15 (1,7). The IGF-IR promoter region is GC-rich, and similar to other housekeeping genes lacks TATA or CCAAT boxes, but contains several sites for binding transcriptional factors such as SP-1, E2F, and early growth response (EGR) proteins (5,8,9). The expression of the IGF-IR is regulated by different physiologic stimuli and may be altered in certain pathologies (e.g., diabetes, cancer). For instance, IGF-IR mRNA is enhanced by growth hormone, follicle stimulating hormone, luteinizing hormone, thyroid hormones, glucocorticoids, and estrogens (9). Moreover, different mitogens (e.g., platelet-derived growth factor, fibroblast growth factor) or oncoproteins (e.g., c-myb, hepatitis B Hbx) can induce IGF-IR transcription. Conversely, IGF-IR expression is downregulated by high concentrations of IGF-II, interferon, antiestrogens, and tumor supressors (e.g., Wilms' tumor or p53 proteins) (4,8,9).

The major IGF-IR 11 kb transcript is translated into a single 1,367 amino acid (aa) (180 kDa) precursor protein, which is then cleaved to form 135 kDa alpha and 90 kDa beta subunits. A mature IGF-IR is a heterotetramer composed of two alpha and two beta subunits linked by disulfide bonds (Fig. 1). The extracellular alpha subunits are responsible for ligand binding. IGF-IR beta subunits, which contain short transmembrane and large intracellular segments, transmit ligand-induced signal (1,7,9). Within the beta subunit, three major domains have been recognized: a tyrosine kinase domain, a juxtamembrane part, and the C-terminus, each containing residues essential for different IGF-IR functions (Fig. 1). Specifically, in the kinase domain, the ATP binding site containing lysine (Lys) 1003 as well as the tyrosine (Tyr) cluster (Tyr 1131, 1135, 1136) are critical for the catalytic activity of the receptor (5,9,10). In the juxtamembrane domain, Tyr 950 flanked by the NPEY motif is required for recruiting major signaling substrates such as insulin receptor substrates (IRS) 1-4 and src/collagen-homology (SHC) proteins (5,6,9,10). The C-terminus contains several residues essential for IGF-I signaling, including Tyr 1250, Tyr 1251, a stretch of serines (Ser) 1280-1283, histidine (His) 1293, Lys 1294, and Tyr 1316. In particular, the region between residues 1229 and 1245 has been found necessary for the association of an adapter GRB10, Tyr 1251 is required for binding a putative substrate p28, Ser 1280-1283 are necessary to sequester an adapter 14-3-3 epsilon, and Tyr 1316 is capable of recruiting either p85 subunit of phosphatidyl inositol-3 kinase (PI-3K) or SHPTP2 phosphatase (5,9,10, Baserga *et al.*, unpublished data). According to recent evidence, Tyr 1251 also appears to be indirectly involved in binding of SHC to the IGF-IR (11).

The IGF-IR shares significant structural homology with the IR. The kinase domains of these receptors are 80–90% identical. Also, Tyr 950 of the IGF-IR has its equivalent, Tyr 960, in the juxtamembrane domain of the IR (7). Importantly, the C-terminal regions of the receptors are quite different, sharing only approximately 40% homology. The equivalents of Tyr 1250 and 1251, Ser 1280–1283, and aa 1293–1301 are not present in the IR. Consequently, it is believed that the differences between biological responses of the IGF-IR and IR are associated with the induction of specialized signaling pathways arising from the C-terminus (1,7,10).

IGF-IR SIGNALING

Upon ligand binding, IGF-IRs cluster and tyrosine kinase is activated leading to autophosphorylation and transphosphorylation of beta subunits (1). Phosphorylation of specific Tyr and Ser residues creates binding sites for IGF-IR signaling substrates. The best known substrates are docking proteins IRS-1 and SHC. Both bind Tyr 950 through their phosphotyrosine binding (PTB) domain (4,9).

IRS-1 is a remarkable effector of the IGF-IR, capable of amplification and diversification of the signal because it can recruit various signaling molecules and induce numerous cellular responses. IRS-1 contains about 20 tyrosine phosphorylation sites which can directly bind signaling molecules equipped with phosphotyrosine binding domains, such as src-homology 2 (SH2) domains. For instance, there are nine YMXM motifs in IRS-1 that can attract the p85 subunit of PI-3K via SH2-type interactions and other domains recruiting SH2-containing adapters GRB2, Nck, and Crk, SHP2 phosphotyrosine phosphatase, and Fyn

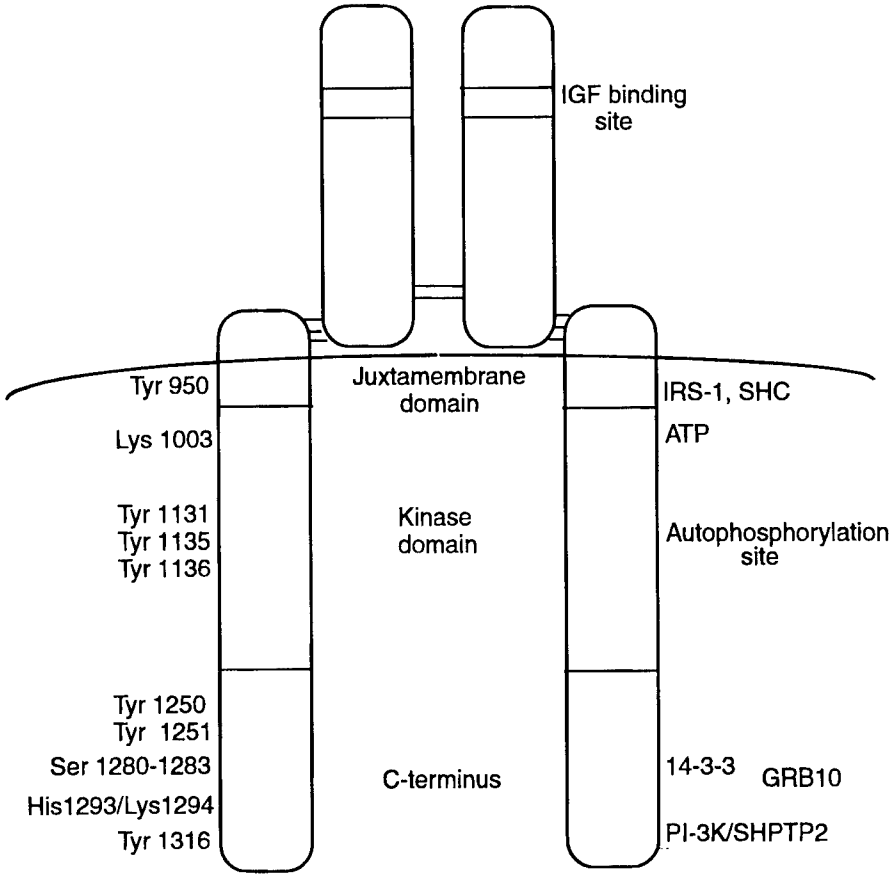

Fig. 1. Structure of the IGF-IR. The key residues involved in IGF-IR signaling are indicated on the left; the signaling elements binding to these regions of the IGF-IR are listed on the right.

kinase. Other partners of IRS-1, such as integrin $\alpha v\beta 3$ or the adapter 14-3-3, associate with the substrate through unknown mechanisms (12).

The major pathway induced by tyrosine phosphorylation of IRS-1 involves PI-3K, whose downstream effectors are Ser/threonine kinases Akt, $p70^{S6}$, and some isoforms of protein kinase C (PKC) (13). PI-3K is involved in the regulation of mitogenesis, metabolism, and actin cytoskeleton rearrangements, and the PI-3K/Akt pathway has recently been recognized as one of the most important signals ensuring cell survival. One of the cellular targets of Akt is a pro-apoptotic protein BAD, which induces cell death when bound to anti-apoptotic proteins Bcl2 or Bcl_{XL}. The phosphorylation of BAD by Akt facilitates its sequestration by 14-3-3 adapters and prevents apoptosis. Another effector of Akt is $p70^{S6}$ kinase which activates expression of cyclin D1 initiating cell cycle progression (13,14).

Association between IRS-1 and the GRB2/SOS complex leads to the stimulation of the classic Ras/MAP cascade of kinases, the pathway that is implicated in a broad array of biological responses, including cell growth and differentiation (12,15). The Ras/MAP pathway can also be induced through Nck or Crk adapters binding to IRS-1 or by GRB2 binding to IGF-IR-associated SHC proteins (6,12,15).

Three additional IRS proteins (IRS-2, -3, and -4) exhibiting different degrees of structural homology to IRS-1 have been cloned. The activities of IRS-1 and IRS-2 appear to partially overlap; for instance, in IRS-1-knock-out mice, IRS-2 substituted for IRS-1 function in stimulating PI-3K and activating glucose metabolism. However, in IRS-1-deficient fibroblasts, only IRS-1, but not IRS-2, reconstituted cell cycle progression (12,13). The functions of IRS-3 and -4 are not well understood. Similarly, the pathways initiated by

the binding of GRB10 or 14-3-3 to the IGF-IR are still obscure.

IGF-IR SIGNAL SPECIFICITY

Because the IGF-IR may regulate many, often contradictory (e.g., growth vs. differentiation), processes, it is of great importance to understand how signal specificity is achieved. Here, I will focus on IGF-IR-dependent survival, mitogenesis, and anchorage-independent growth, the most studied IGF effects. The current view is that the IGF-I response is dictated by the engagement of different sets of intracellular pathways. Which pathways are stimulated depends on (i) the number of activated receptors on the cell surface; (ii) the availability of signaling substrates and receptor-substrate binding sites; (iii) the abundance and activity of negative regulators such as phosphatases; and (iv) extracellular context, e.g., ligand availability or extracellular matrix (ECM) components and their interaction with cells.

The first point is best illustrated by the work of Rubini *et al.* (16) and Reiss *et al.* (17), who analyzed the relationship between the number of IGF-IRs expressed on the cell and IGF-I-induced biological response. While activation of $2–10 \times 10^3$ receptors stimulates tyrosine phosphorylation of IRS-1 as well as activation of an early response gene *c-myc*, it is not sufficient for SHC phosphorylation or the entry of cells into the cell cycle. With 1.5×10^4 receptors, the cells progress through the S phase, but they are not able to complete cell division, and their survival ability under anchorage-independence is minimal. The increase of receptor expression to $2.2–6.0 \times 10^4$ receptors/cell ensures phosphorylation of the major substrates, full mitogenic response, and good survival, but produces only a weak transforming activity (measured by growth in soft agar). Activation of more than 1×10^5 IGF-IRs provides signal strong enough to activate both IRS-1 and SHC signaling pathways, stimulate cell division, and support robust transformation (16,17). A direct relationship between the number of stimulated IGF-IRs and cell survival and/or tumorigenesis in animal models has been documented by the Baserga and LeRoith laboratories (18,19). For instance, NIH 3T3 mouse fibroblasts expressing 1.9×10^5 IGF-IR/cell form tumors in nude mice, while fibroblasts with lower IGF-IR levels (1.6×10^4) are nontumorigenic. In addition, the latency of tumor formation *in vivo* was reduced with high doses (4–10 mg/kg) of endocrine IGF-I, suggesting that chronic stimulation of a high number of IGF-IRs was critical for the onset of tumorigenesis (19).

A mutational analysis has been performed to determine whether various functions of the IGF-IR are induced by overlapping or distinct pathways. In the studies of Baserga *et al.*, different mutant IGF-IRs have been expressed in R-minus cells (derived from IGF-IR knock-out mice), which allowed the analysis of signaling pathways of the mutants without interference from the endogenous wild-type IGF-IRs (10). This work was complemented by O'Connor *et al.* who studied mutant receptors expressed in either hematopoietic IRS-1-negative FL5.12 cells or apoptosis-prone Rat-1/Myc fibroblasts, and by LeRoith *et al.* who used NIH 3T3 fibroblasts for the analysis (5,6). All studies demonstrated that a mutation in the ATP binding site produced "dead" receptors incapable of signal transmission. Replacement of all three Tyr 1131, 1135, and 1136, or Tyr 1136 alone, with phenylalanine produced a receptor that was not mitogenic or transforming, but it still induced an efficient survival signal. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth. Tyr 950 in the IGF-IR juxtamembrane domain was found necessary for IRS and SHC association, and for induction of mitogenic and transforming activity. Interestingly, however, the IGF-IR/Tyr 950 mutant transmitted anti-apoptotic signaling. This finding indicates that in addition to the classic IRS-1-dependent PI-3K/Akt pathway, other survival pathway(s) emanate from the IGF-IR (5,6,9,10).

Deletion of the entire C-terminus at aa 1229 produced a receptor that retained normal mitogenic function but was totally lacking transforming potential (20). Subsequent detailed studies with mutant IGF-IRs expressed in R-minus cells mapped the "transforming domain" between residues 1245 and 1310, with Tyr 1251, Ser 1280–1283, His 1293, and Lys 1294 required for transformation (10). Importantly, this region does not have an exact counterpart in the IR. Indeed, the expression of the IR or a chimeric IGF-IR containing an IR C-terminus did not support soft agar growth of R-minus cells (10). Notably, the IGF-IR transforming signal appears to be truly unique, at least in mouse fibroblasts, as overexpression of various growth factor receptors, signaling molecules or oncogenes (except for v-src), singly or in combination, did not restore transformation in R-minus cells, while the IGF-IR did (3,21). The mediators of the IGF-IR transforming pathway are not yet known, but the adapters 14-3-3 and

GRB10, which bind to the C-terminus, could be involved.

Interestingly, the IGF-IR C-terminus also appears to play a unique role in survival signaling. Mutants with a deleted C-terminus (at residues 1229 or 1245) retained or even amplified anti-apoptotic function, while single mutations in Tyr 1251, His 1293, and Lys 1294 reduced survival (5). Consequently, it has been suggested that the C-terminus is an intrinsic inhibitory domain of the IGF-IR, while the residues Tyr 1251, His 1293, Lys 1294 act as neutralizers of this pro-apoptotic function. Indeed, expression of the C-terminal 108 aa as a membrane-targeted protein resulted in induction of apoptosis, and mutations in Tyr 1250/1251 and His 1293/Lys 1294 abrogated this cytotoxic activity (5).

To summarize, IGF-IR signals required for mitogenesis, transformation, and survival are distinct but partially overlap. For instance, no transforming activity is seen in the absence of mitogenic activity. Transformation also seems to have some common pathways with IGF-dependent survival. However, cell survival can be induced by a weak signal which is not sufficient for mitogenesis or transformation, while transformation requires strong IGF-IR activation and induction of specific signals originating at the C-terminus.

The pathways mediating non-growth IGF-I responses such as cell-cell or cell-substrate interactions are less well characterized. Our preliminary data indicate that in epithelial cells, intercellular adhesion requires the tyrosine kinase domain as well as the C-terminus of the IGF-IR, and depends on SHC but not on IRS-1 signaling (4,22, Surmacz *et al.*, unpublished data). IGF-I-induced motility and reorganization of actin cytoskeleton involves PI-3K and SHC activities, and modification of proteins associated with focal adhesions (22,23).

It is known that the IGF-IR response may be cell-type specific (24). One mechanism ensuring such specificity is the availability of intracellular signaling intermediates. For instance, with the same cellular content of the IGF-IR, downregulation of IRS-1 expression inhibits cell growth, transformation, and results in cell death, while amplification of IRS-1 sensitizes cells to low concentrations of IGF-I and enhances anchorage-independent growth (22,25,26). On the other hand, overexpression of SHC does not improve IGF-I-dependent growth, but inhibition of SHC expression inhibits cell growth, transformation, and to a lesser extent, cell survival (22,27).

Finally, the extracellular context plays a role in IGF-I response, for instance, survival and growth of cells adhering to a proper substrate is mediated through the IRS-1 pathway, while the same pathway is much less important in IGF-I-dependent protection from apoptosis due to anchorage-independence (22,28,29).

Requirement for IGF-IR in Proliferation, Transformation, and Survival of Breast Cancer Cells

The critical role of the IGF-IR in breast cancer growth, survival, and transformation has been well documented *in vitro* and in animal models (Table I) (4). Reducing ligand availability by excess IGF-BP1 or exposure to suramin blocked IGF-IR activation and limited breast cancer cell proliferation. Furthermore, inhibiting the expression of the IGF-IR with an antisense-IGF-IR RNA, or its function with anti-IGF-IR antibodies or dominant-negative mutants, resulted in growth inhibition and reduced transforming potential (4). Our studies with MCF-7 breast cancer cell lines expressing antisense-IRS-1 or antisense-SHC RNAs (MCF-7/anti-IRS-1 or MCF-7/anti-SHC cells) demonstrated that both IRS-1- and SHC-dependent signals are necessary for cell proliferation and transformation (22). The critical role of IRS-1 (but not IRS-2) and IRS-1 downstream pathways—Ras/MAP and PI-3K in the growth of estrogen receptor (ER)-positive breast cancer cells has recently been confirmed by the Yee laboratory (30). Using dominant-negative IGF-IRs lacking the C-terminus, we demonstrated that in breast tumor cells, as in fibroblasts, the C-terminal portion is essential for transformation *in vitro* and tumorigenesis *in vivo* (4).

Dunn *et al.* have shown that activation of the IGF-IR protects breast cancer cells from apoptosis induced by various therapeutic agents, serum deprivation and irradiation (31). Our results with MCF-7 cells in which IRS-1 has been downregulated by either antisense-IRS-1 oligonucleotides, expression of antisense-IRS-1 RNA, or antiestrogen treatment suggest that the IRS-1/PI-3K signal is required for IGF-IR-induced survival (22,25,32).

Amplification of IGF-IR Signaling and Anchorage-Dependent and -Independent Growth of Breast Cancer Cells

Further understanding of IGF-IR function in breast cancer pathobiology stemmed from studies of

Table I. IGF-IR Function in Breast Cancer

Signaling molecule	Function in breast cancer	
	Experimental models	Tumors
IGF-IR	elevated in ER-positive breast cancer cells; stimulates proliferation; counteracts apoptotic effects of anti-tumor drugs; improves 3-D growth and survival; regulates cell-substrate connections; required for anchorage-independent growth *in vitro* and tumorigenesis and metastasis in animal models.	correlates with the ER status; elevated in primary tumors; high levels correlate with radio-resistance and recurrence at the primary site; usually co-expressed with markers of better overall prognosis.
IRS-1	elevated in ER-positive breast cancer cells; required for anchorage-dependent and independent growth; critical for survival; high levels induce estrogen-independence and antiestrogen-resistance.	correlates with shorter DFS in ER-positive primary tumors.
SHC	required for proliferation, anchorage-independent growth, migration, and cell-cell adhesion; high levels improve adhesion to fibronectin.	?

cells with amplified IGF-IR signaling. In order to correlate the strength of the IGF signal with the progression towards a more neoplastic phenotype, we developed a series of MCF-7-derived cell lines overexpressing different levels of either the IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells), or SHC (MCF-7/SHC cells) (25,27,29).

Overexpression of the IGF-IR (8–50-fold) was paralleled by enhanced IGF-IR tyrosine kinase activity and hyperphosphorylation of IRS-1, even in the absence of exogenous IGF-I. Compared to the parental cells, all MCF-7/IGF-IR clones exhibited enhanced autocrine growth in serum-free medium and improved growth responsiveness to low concentrations of IGF (0.1–1.0 ng/ml), especially in the presence of 10 nM estradiol (E2). With higher doses of IGF-I (4–50 ng/ml), the synergistic effect was not seen and the maximal mitogenic effect was achieved with IGF-I alone (29). Similar results were described by Daws *et al.*, who independently developed IGF-IR overexpressing MCF-7 clones (33). Interestingly, we as well as others noticed that high doses of IGF-I (20–50 ng/ml) combined with 10 nM E2 inhibited MCF-7/IGF-IR cell growth, especially in the clones with the highest IGF-IR levels (29,33).

Anchorage-independent growth of MCF-7/IGF-IR cells treated with E2 was slightly elevated relative to the parental cells, but this effect of IGF-IR overexpression was not present in cells treated with both E2 and IGF-I or cultured in serum-containing medium (29,33).

In contrast with the modest effects of amplified IGF-IR, overexpression of IRS-1 (1.5–9-fold) produced marked changes in the growth phenotype (25). In MCF-7/IRS-1 cells, proliferation was enhanced under all conditions studied (serum-free and serum-containing medium, or serum-free medium with 20 ng/ml IGF); the addition of E2 never inhibited the growth. Also, MCF-7/IRS-1 cells exhibited greatly a enhanced potential for growth in soft agar, especially in the presence of high (200–400 ng/ml) doses of IGF-I. Remarkably, this IGF-dependent transformation was further potentiated with E2. The above effects were correlated with the cellular levels of IRS-1 and the extent of IRS-1 tyrosine phosphorylation (25).

Amplification of SHC in MCF-7 cells (two–sevenfold) did not alter growth properties under standard monolayer or anchorage-independent conditions, but it amplified cell-substrate interactions on fibronectin (27).

IGF-IR/ER Cross-Talk

In hormone-dependent breast cancer cells, ER and the IGF-IR are co-expressed and E2 acts in synergy with IGF-I to stimulate proliferation (4). The effects of E2 are mediated in part via sensitization of cells to IGF action. E2 treatment up-regulates IGF-IR mRNA and protein levels by two–tenfold, reflected in enhanced IGF-IR tyrosine phosphorylation (4,34,35). Furthermore, E2 significantly (two–fivefold) stimulates the expression of IRS-1 in different ER-positive cell lines, and the extent of this stimulation depends on the cellular ER content (35, Surmacz *et al.*, unpublished data). Of note, E2 action appears to be at least partially specific to the IGF-IR/IRS-1 pathway since it does not modulate SHC levels (36).

Importantly, various antiestrogens such as Tamoxifen (Tam) and its derivatives, droloxifene, and

pure antiestrogens ICI 164,384 and ICI 182,780 inhibit IGF-IR-dependent proliferation (4,32,36–38). We demonstrated that at the molecular level, the anti-IGF-IR actions of Tam and ICI 182,780 are accomplished by downregulation of IRS-1/PI-3 kinase signaling (32,36). Specifically, growth arrest and apoptosis resulting from antiestrogen treatment were associated with continuos suppression of IRS-1 mRNA and protein expression, reflected by reduced IRS-1 tyrosine phosphorylation, decreased IRS-1/PI-3K binding and reduced PI-3K activity (32,36). These anti-IRS-1 effects of ER antagonists were partially reversed in the presence of IGF-I (36).

Antiestrogens also inhibit IGF-IR expression and tyrosine phosphorylation (by 30–50%) but only in the presence of IGF-I (32,36). In the absence of IGF-I, Tam and ICI 182,780 enhance IGF-IR phosphorylation, which suggest that the drugs may act through modulation of IGF-I-dependent phosphatases. Indeed, the involvement of tyrosine phosphatases LAR and FAP-1 in antiestrogen inhibition of IGF-dependent growth has been demonstrated by the Vignon laboratory (39). Interestingly, in different antiestrogen-treated cell lines, SHC expression or signaling were not altered, while SHC tyrosine phosphorylation was increased in Tam- but not in ICI 182,780-arrested cells (32,36).

Because E2 upregulates IGF-IR signaling, it has been postulated that amplification of the IGF-IR or its key signaling substrates may lead to estrogen-independence. In agreement with this hypothesis, MCF-7/IRS-1 cells exhibited reduced estrogen requirements for growth and transformation, and were not inhibited by E2 alone or in combination with of IGF-I (25). Interestingly, however, such estrogen-independence has not been detected in MCF-7/IGF-IR cells (29,33). These cells still appear to remain under ER control as their growth is restrained by high doses of E2 in the presence or absence of IGF-I. This finding suggests the existence of a negative growth regulatory loop which is not operative in MCF-7/IRS-1 cells and may be triggered by hyperactivation of IGF-I signaling pathways not involving IRS-1.

The role of amplified IGF-IR signaling in the development of antiestrogen-resistance is of particular interest. We and others have shown that overexpression of different IGF-IR signaling elements did not affect ER content (25,29,33). However, MCF-7/IRS-1 clones with very high IRS-1 levels (9 or 12-fold overexpression with respect to MCF-7 cells) exhibited resistance to ICI 182,780, confirming that the IRS-1 pathway is an essential target for antiestrogens and suggesting that overexpression of IRS-1 in tumors may hinder antiestrogen therapy (36). Interestingly, in contrast with IRS-1, overexpressed IGF-IRs (50-fold) or SHC (fivefold) did not alter antiestrogen sensitivity in MCF-7 cells (36).

IGF-IR-DEPENDENT CELL-CELL AND CELL-SUBSTRATE INTERACTIONS IN BREAST CANCER CELLS

Breast cancer cells, like other polarized epithelial cells, are governed by cell-cell and cell-substrate interactions. The regulation of these processes by growth factors is now being increasingly recognized. We studied intercellular interactions of MCF-7 cells and their derivatives with modified IGF-IR signaling. We found that overexpression of the IGF-IR greatly enhanced aggregation of cells in three-dimensional (3-D) culture (29). Specifically, when plated on Matrigel, MCF-7/IGF-IR cells formed large spheroids (150–300 μm in diameter) surviving or even proliferating for up to 20 days, while the parental MCF-7 cells formed smaller clusters (50 μm) which disaggregated and died after 7 days of culture. Similar stimulation of cell-cell adhesion has been described in IGF-I treated MCF-7 and MCF-7/6 cells as well as in MCF-7 cells constitutively secreting IGF-I (4). Our subsequent research demonstrated that enhanced cell-cell adhesion is IGF-I-specific as it cannot be induced by physiologic concentrations of EGF, IGF-II or insulin (4). The mechanism of this phenomenon is still not clear. We have shown that the IGF-IR co-localizes with an adherens junction protein E-cadherin and co-precipitates with E-cadherin, α-catenin, and β-catenin (4,29). In addition, we obtained preliminary data suggesting that the IGF-IR stimulates the expression of a junction protein ZO-1, thereby strenghtening the α-catenin/ZO-1/F-actin connections (Surmacz *et al.*, in preparation). The signals required for cell-cell adhesion depend on IGF-IR tyrosine kinase activity and the presence of the C-terminus (4, Surmacz *et al.*, in preparation). We also observed that cell-cell adhesion is reduced in MCF-7/anti-SHC, but not MCF-7/anti-IRS-1 cells, which points to SHC as a putative mediator of IGF-induced aggregation (22).

The enhanced intercellular connections and improved survival of IGF-IR overexpressing cells may contribute to their tumorigenic activity *in vivo*. Indeed, when MCF-7/IGF-IR cells were injected into mam-

mary fat pad of nude mice, they formed tumors after 8 weeks, while the parental MCF-7 cells or MCF-7 clones expressing an IGF-IR with a C-terminal truncation were non-tumorigenic (Surmacz *et al.*, in preparation).

Cell-substrate adhesion and migration of epithelial cells is also regulated by IGF-I. For instance, depolarization of MCF-7 and MCF-7/IGF-IR cells and induction of cell migration can be achieved with a 4 hour treatment with 50 ng/ml IGF-I. The initial stages of this process are associated with transient dephosphorylation of the focal adhesion proteins FAK, paxillin and p130 Cas (23). IGF-IR pathways involved in the regulation of breast cancer cell motility are still quite obscure, but we observed reduced migration of MCF-7 cells with impaired SHC or PI-3K signaling (22,23).

Obviously, extracellular cell context may dictate whether cellular response to IGF-I involves increased cell-cell adhesion or enhanced migration. Increased intercellular adhesion may be seen in cells expressing low levels of integrins necessary for attachment to a given ECM substrate, whereas enhanced migration occurs when the cells interact well with a substrate, or produce sufficient amounts of their own ECM (23,28).

IGF-IR Signaling in Breast Tumors

Studies on IGF-IR expression in breast tumors and its correlation with other host or tumor parameters are very limited (4). Moreover, the interpretation of the available data is complicated by the fact that different techniques were used to assess the IGF-IR levels. The most frequently performed IGF-I binding assay is inherently inaccurate due to the interaction of IGF with membrane IGFBPs, often resulting in overestimation of the number of the IGF-IR (4). To circumvent this problem, the expression of IGF-IR in tumor samples was examined with anti-IGF-IR antibody-based techniques (radioimmunoassay or immunocytochemistry) (40,41). Despite the differences in experimental approach, in all large series studies (>100 cases) the IGF-IR has been detected in a majority of breast tumor samples (4,42,43).

Most important, IGF-IR levels have been found to be elevated (up to 14-fold) in primary breast cancer compared to non-malignant tumors or normal epithelium (40–43) (Table I). The mechanism of the common IGF-IR overexpression in breast cancer is not clear, but it does not appear to be associated with IGF-IR gene amplification since this event was reported in only 2% of cases analyzed (44). Recent data indicate that IGF-IR overexpression may be related to derepression of IGF-IR transcription due to aberrant expression of the tumor supressor protein p53 (40,45,46). Overexpression of the IGF-IR in tumors has been found to be associated with hyperactivation of the tyrosine kinase (up to sixfold), and correlated with radio-resistance and tumor recurrence at the primary site (40,41). High IGF-IR levels in primary tumors have been reported as predictors of shorter disease-free survival, but data on the prognostic value of the IGF-IR for overall survival are conflicting (4,41).

Importantly, not only the IGF-IR, but also IRS-1 has been found to be overexpressed in a fraction of primary breast tumors (35,47). High levels of IRS-1 correlated with shorter disease-free survival in ER-positive tumors (35). The mechanism of IRS-1 overexpression is not known, but it could be associated with E2 or IGF activity since both mitogens are known to stimulate IRS-1 transcription and both can be found (often at superphysiological concentrations) in breast tumors.

Attempts to correlate IGF-IR expression with other host or tumor variables showed a positive link between the IGF-IR and ER status (4,42,43). In addition, frequent co-expression of the IGF-IR and IR has been shown (42). Co-expression of these structurally homologous receptors leads to the formation of functional hybrids which bind IGF-I with high affinity, and thereby amplify the IGF-I signal (48).

In several large series analyses, no significant correlations were found between IGF-IR expression and menopausal status (42), body weight (42), tumor size (42,43), tumor grade (42,49,50), histological type (42,43), node status (42,43,49,50), or EGFR status (49), and the link with the progesterone receptor (PgR) status is uncertain (4,42, 43,49–51). However, because most of these associations were established based on IGF-I binding assays, they should be re-assessed using more accurate techniques of IGF-IR measurement before any firm conclusions can be drawn.

The expression of IRS-1 correlated with ER levels but not with other parameters such as age, tumor size, or PgR status (35,47). The levels of another IGF-IR substrate, SHC, are similar in aggressive and more differentiated breast cancer cell lines, but its activity (tyrosine phosphorylation) in cell lines and tumors reflects the levels of oncogenic kinases ERB2 or c-src

(27,52,53). SHC association with IGF-IR in breast tumors has not been studied.

The Unclear Role of the IGF-IR in Breast Cancer Progression

The experimental and clinical evidence points to the fact that the IGF-IR may be important in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages, including metastasis, is still obscure.

Especially intriguing is the fact that, whereas the IGF-IR has been found to be overexpressed in primary tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease. For instance, Pezzino *et al.* assessed IGF-IR status in two patient subgroups, representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population and found a highly significant correlation between IGF-IR expression and better prognosis (42). Similar conclusions were reached by Peyratt and Bonneterre (43). Therefore, it has been proposed that like the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to have a worse prognosis than node-negative/IGF-IR-positive tumors (49). In addition, quite rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter disease-free survival (48).

In breast cancer cell lines, a hormone-dependent and less aggressive phenotype correlates with a good expression of the IGF-IR and IRS-1 (29,35). In contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and often do not respond to IGF-I with growth (54,55). Similarly, IRS-1 levels are downregulated in a majority of these cell lines (35,55). Despite this "IGF-IR-reduced phenotype", metastatic cell lines appear to depend on the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration *in vitro* and tumorigenesis *in vivo*, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal models (56–58). Whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the survival function of the IGF-IR is under investigation in our laboratory.

CONCLUSIONS

Over the past few years much has been learned about the function of the IGF-IR in the process of tumorigenesis. Clearly, IGF-IR-mediated survival and transformation are key factors affecting tumor development. In primary breast cancer, high levels of the IGF-IR may promote survival and proliferation, counteracting cytotoxic or cytostatic effects of drugs or radiation. The mechanism of this IGF-I action includes strengthening intercellular connections, amplification of anti-apoptotic signals, and sensitization of cells to low concentrations of IGFs and E2. Therefore, targeting the IGF-IR, especially the IGF-IR/IRS-1 pathway, should help in eradicating primary tumor cells.

The importance of the IGF-IR in metastatic breast disease is still not clear. It is possible that the IGF-IR has a role in cell spread, functioning primarily as an anti-apoptotic, and possibly a motogenic factor. Unquestionably, further understanding of IGF-IR function in metastatic cells will be critical in creating successful anti-IGF-IR therapies for late stages of breast cancer.

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SHC–α5β1 Integrin Interactions Regulate Breast Cancer Cell Adhesion and Motility

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The oncogenic SHC proteins are signaling substrates for most receptor and cytoplasmic tyrosine kinases (TKs) and have been implicated in cellular growth, transformation, and differentiation. In tumor cells overexpressing TKs, the levels of tyrosine phosphorylated SHC are chronically elevated. The significance of amplified SHC signaling in breast tumorigenesis and metastasis remains unknown. Here we demonstrate that seven- to ninefold overexpression of SHC significantly altered interactions of cells with fibronectin (FN). Specifically, in human breast cancer cells overexpressing SHC (MCF-7/SHC) the association of SHC with α5β1 integrin (FN receptor) was increased, spreading on FN was accelerated, and basal growth on FN was reduced. These effects coincided with an early decline of adhesion-dependent MAP kinase activity. Basal motility of MCF-7/SHC cells on FN was inhibited relative to that in several cell lines with normal SHC levels. However, when EGF or IGF-I was used as the chemoattractant, the locomotion of MCF-7/SHC cells was greatly (approx fivefold) stimulated, while it was only minimally altered in the control cells. These data suggest that SHC is a mediator of the dynamic regulation of cell adhesion and motility on FN in breast cancer cells. © 1999 Academic Press

Key Words: **SHC; α5β1 integrin; fibronectin; motility; breast cancer.**

INTRODUCTION

The ubiquitous SH2 homology/collagen homology (SHC) proteins (p46, p52, and p66) are overlapping SH2-PTB adapter proteins that are targets and downstream effectors of most transmembrane and cytoplasmic tyrosine kinases (TKs) [1, 2]. Consequently, overexpression of $p52^{SHC}$ and $p46^{SHC}$ (referred to as SHC hereinafter) amplifies various cellular responses; for instance, it induces mitogenic effects of growth factors in NIH 3T3 mouse fibroblasts and myeloid cells [1, 3], stimulates differentiation in PC12 rat pheochromocytoma [4], and augments hepatocyte growth factor (HGF)-induced proliferation and migration in A549 human lung adenocarcinoma [5]. Overexpressed SHC is oncogenic in NIH 3T3 mouse fibroblasts, but amplification of $p66^{SHC}$ isoform does not induce transformation [1, 6, 7] and may even inhibit growth pathways [8]. Importantly, increased tyrosine phosphorylation of SHC, which has been noticed in different tumor cell lines, is a marker of receptor or cytoplasmic TKs overexpression [2]. In breast cancer, for instance, SHC is hyperphosphorylated in cells overexpressing ERB-2 and c-Src [9, 10]. Whether such amplification of SHC signaling contributes to the development of a more aggressive phenotype of breast tumor cells remains unknown.

The effector pathways downstream of SHC are partially known. Upon tyrosine phosphorylation by TKs, SHC associates with the GRB2/SOS complex and subsequently stimulates the canonical Ras–MAPK (p42 and p44 mitogen-activated protein kinases) signal transduction cascade [1, 6, 7]. SHC/GRB2 binding and the activation of Ras are prerequisites for SHC-induced mitogenesis and transformation in NIH mouse fibroblasts [6]. In addition, SHC has been described as associating with adapters Crk II [11] and GRB7 [12] as well as with a signaling protein p145 [13, 14] and PEST tyrosine phosphatase [15] in various experimental systems. However, SHC pathways incorporating these signaling intermediates and their biological significance are not well understood.

There is substantial evidence suggesting that in addition to its role in mitogenesis and transformation, SHC regulates nongrowth processes, such as cell adhesion and motility. For instance, overexpressed SHC improved motility in HGF-stimulated melanoma cells [5], and downregulation of SHC reduced epidermal growth factor (EGF)-dependent migration in MCF-7 breast cancer cells [16]. SHC was also essential for

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kidney epithelial cell scattering mediated by the receptors c-met, c-ros, and c-neu [17]. The mechanisms by which SHC regulates cell adhesion and motility are still obscure.

In several cell types (Jurkat, HUVEC, MG-63, and A431 cells), SHC couples with certain ECM receptors, specifically with the integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha6\beta4$, but not with $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ [18]. Association of SHC with integrins may result in phosphorylation of SHC by integrin-associated TKs (Fyn, other Src-like kinases, FAK, or FAK-associated kinases) and subsequent activation of MAPK [18–21]. The biological significance of integrin-stimulated MAPK activity is not well understood. However, recent data indicated that it positively regulates cell growth and survival [18, 22], but is not essential for cell migration [23].

This work addresses the consequences of amplified SHC signaling on proliferation, transformation, adhesion, and motility in breast cancer MCF-7 cells. In these cells, SHC is an important intermediate of different signaling pathways. Growth factors present in serum, such as IGF-I and EGF, can induce SHC through their cognate receptors [1, 16, 24]. Estrogens (also contained in serum) may elevate tyrosine phosphorylation of SHC via cytoplasmic TKs of the Src family [25]. In addition, SHC can be stimulated by cytoplasmic TKs as a result of cell spreading on ECM [18]. MCF-7 cells express several integrin receptors: $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, and $\alpha v\beta5$ [26]. Of those, $\alpha5\beta1$, a FN receptor, is known to associate with SHC, while $\alpha2\beta1$ and $\alpha3\beta1$ are not SHC binding proteins [18].

The interactions of cells with FN have been reported to influence or control different processes regulating the behavior of cancer cells, namely cell migration, invasion, and metastasis as well as survival and proliferation [27]. The exact role of $\alpha5\beta1$ FN receptors in tumor progression is not clear. It has been shown that extracellular matrix recognition by $\alpha5\beta1$ integrin is a negative regulator of cell growth and may be lost in some tumor cells [28]. In agreement with this, overexpression of $\alpha5\beta1$ integrin and improved cell spreading on FN can reduce cell growth and transformation *in vivo* and reverse tumorigenicity *in vitro* [29, 30]. On the other hand, FN receptors may play a role in later stages of tumor progression since blocking $\alpha5\beta1$ integrin abrogated cell spread in experimental breast metastasis [31]. The importance of SHC signaling in the interactions of breast cancer cells with FN has not been studied and is a subject of this work.

MATERIALS AND METHODS

Cell lines and cell culture conditions. MCF-7 cells are estrogen receptor positive cells of a low tumorigenic and metastatic potential. The growth of MCF-7 cells is controlled by estrogens, such as estradiol (E2), and growth factors, such as IGF-I and EGF [32, 33]. MCF-7 cells express several integrins, including $\alpha5\beta1$ (FN receptor), $\alpha2\beta1$ (collagen, COL receptor), $\alpha3\beta1$ (COL/FN/laminin 5 receptor), and $\alpha v\beta5$ (vitronectin receptor) [26].

MCF-7/SHC clones 1 and 9 are MCF-derived cells stably transfected with the expression plasmid pcDNA3/SHC containing a human SHC cDNA encoding $p55^{SHC}$ and $p47^{SHC}$. The clones expressing the transgene were selected in 2 mg/ml G418, and the levels of SHC expression in 20 G418-resistant clones were determined by Western blotting (WB) in whole cell protein lysates, as described below.

As control cells, we used several MCF-7-derived clones with modified growth factor signaling pathways: specifically, MCF-7/IRS-1, clones 3 and 18, which are MCF-7 cells overexpressing insulin receptor substrate 1 (IRS-1) [32]; MCF-7/IGF-IR, clone 17, which is an MCF-7-derived clone overexpressing the insulin-like growth factor 1 receptor (IGF-IR) [33]; and MCF-7/anti-SHC, clone 2, with SHC levels decreased by 50% due to the stable expression of an anti-SHC RNA [16].

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 μg/ml G418. In the experiments requiring E2- and serum-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM FeSO4, and 2 mM L-glutamine (referred to as PRF–SFM).

Monolayer growth. Cells were plated at a concentration 1.5–2.0 $\times$ 10^5 in six-well plates in the growth medium; the following day (day 0), the cells at approximately 50% confluence were shifted to PRF–SFM containing 1 or 20 ng/ml IGF-I or 1 or 10 ng/ml EGF. After 4 days, the number of cells was determined by direct counting.

Anchorage-independent growth. Transforming potential of the cells (anchorage independence) was measured by their ability to form colonies in soft agar, as previously described [32]. The cells, 1 $\times$ 10^3/35-mm plate, were grown in a medium solidified with 0.2% agarose. The solidified medium contained either (i) DMEM:F12 supplemented with 10% FBS or 5% CS or (ii) PRF–SFM with 200 ng/ml IGF-I, 50 ng/ml EGF, or 200 ng/ml IGF-I plus 50 ng/ml EGF. After 21 days of culture, the colonies greater then 100 μm in diameter were counted using an inverted phase–contrast microscope.

Adhesion on FN or COL. Cells synchronized for 24 h in PRF–SFM were seeded in 60-mm plates coated with FN (50 μg/ml) or COL (20 μg/ml). Before the experiment, the plates were blocked with 3% BSA for 3 h at 37°C and then washed once with PBS. To inactivate $\alpha5\beta1$ integrin, the cells were incubated with a blocking $\alpha5\beta1$ Ab 10 μg/ml (Chemicon) for 30 min before plating. Cell morphology was recorded using an inverted phase–contrast microscope with a camera. Percentage of nonadherent cells was determined by counting the number of floating cells vs. the number of cells originally inoculated in the plate.

Growth on FN. Cells (0.5 $\times$ 10^5/ml) were seeded in 12-well plates coated with FN (50 μg/ml) in normal growth medium with or without EGF (10, 50, or 100 ng/ml) or IGF-I (20 or 100 ng/ml). The cells were counted after 4 days of culture.

Motility assay. Motility was tested in modified Boyden chambers containing porous (8-mm) polycarbonate membranes. The undersides of membranes were coated with either 20 μg/ml COL IV or 50 μg/ml FN, as described by Mainiero *et al.* [34]. According to this protocol, collagen (COL) or FN covered not only the underside of the membrane, but also diffused into the pores where cell contact with ECM was initiated. Synchronized cells (2 $\times$ 10^4) suspended in 200 μl of PRF–SFM were plated into upper chambers. Lower chambers contained 500 μl of PRF–SFM with EGF (1 and 10 ng/ml) or IGF-I (20 ng/ml). After 12 h, the cells in the upper chamber were removed, while the cells that migrated to the lower chamber were fixed and stained in Coomassie blue solution (0.25 g Coomassie blue/45 ml water/45 ml methanol/10 ml glacial acetic acid) for 5 min. After that, the chambers were washed three times with H_2O. The cells that migrated to the lower chamber were counted under the microscope as described before [16].

Immunoprecipitation and Western blotting. Proteins were obtained by lysis of cells with a buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, 0.2 mM Na_3VO_4, 1% PMSF, 1% aprotinin. The expression of SHC in transfectants and the parental cells was assessed in 50 μg of total cell lysate using an anti-SHC monoclonal antibody (mAb) (Transduction Laboratories). Alternatively, SHC was detected by immunoprecipitation (IP) from 250–1000 μg (specific amounts are given under the figures) of protein lysate with an anti-SHC polyclonal antibody (pAb) (Transduction Laboratories), followed by WB with an anti-SHC mAb (Transduction Laboratories). Tyrosine phosphorylation of SHC was measured by WB using an anti-phosphotyrosine mAb PY20 (Transduction Laboratories). The levels of $\alpha5\beta1$ integrin were assessed in 1 mg of protein lysate by IP with an anti-$\alpha5\beta1$ pAb (Chemicon) and WB using an anti-$\beta1$ mAb (Chemicon). The amounts of integrin-associated SHC were measured in $\alpha5\beta1$ integrin immunoprecipitates with an anti-SHC pAb (Chemicon). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

MAPK activity. The phosphorylated forms of p42 and p44 MAPK were identified by WB in 50 μg of whole cell lysates with an anti-phospho-MAPK (Thr202/Tyr204) mAb (New England Biolabs). The total levels of MAPK were determined with an anti-MAPK pAb (New England Biolabs). Adhesion-induced MAPK activity was assessed in cells plated either on COL IV or FN and then lysed at different times after plating (0–24 h). To determine EGF-induced MAPK activity, the cells were plated on different substrates, allowed to attach for 1 h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0–24 h) of the treatment and MAPK activity was measured as described above.

Statistical analysis. The results of cell growth experiments were analyzed by Student t test.

RESULTS

Basal and growth factor-induced SHC tyrosine phosphorylation is increased in MCF-7/SHC cells. To investigate the implications of increased SHC signaling in breast cancer cells, we developed several MCF-7-derived clones stably overexpressing $p46^{SHC}$ and $p52^{SHC}$. Of 20 G418 resistant clones, 7 exhibited SHC overexpression, as determined by WB (data not shown). Two representative clones, MCF-7/SHC, 1 and MCF-7/SHC, 9, with a seven- and ninefold SHC amplification, respectively, were selected for subsequent experiments (Fig. 1). The greater amount of SHC in these clones was reflected by increased levels of SHC tyrosine phosphorylation, which was evident in both continuously proliferating (Fig. 1A) and EGF-stimulated cultures (Fig. 1B). The extent of SHC tyrosine phosphorylation roughly corresponded to the cellular levels of the protein (Fig. 1A and B).

Overexpression of SHC has minimal effects on cell growth on plastic and does not enhance transformation in soft agar. The significant hyperactivation of SHC in MCF-7/SHC cells suggested that growth properties of these cells might have been altered. First we determined that in serum-containing medium or PRF–SFM, the growth rate of MCF-7/SHC clones was comparable to that of the parental cells or other cell lines with normal SHC levels (data not shown). Next, we studied mitogenic response to EGF and IGF-I, growth factors which stimulate tyrosine phosphorylation of SHC [1, 24] and require SHC for their growth action [16]. We found that relative to MCF-7 cells, MCF-7/SHC clones exhibited only moderately (20–40%) enhanced growth response to IGF-I or EGF (Fig. 1C). Under the same conditions, the proliferation of a control clone MCF-7/anti-SHC, 2 was substantially (at least by 50%) reduced (Fig. 1C), as we demonstrated previously [16].

Since SHC is oncogenic when overexpressed in NIH mouse fibroblasts [1], we assessed transforming potential of MCF-7/SHC clones in soft agar assay. Despite significant SHC overexpression in these cells, in several repeat experiments and under different growth conditions used, anchorage-independent growth of MCF-7/SHC cells was never enhanced relative to that seen in MCF-7 cells (Table 1). In the same assay, MCF-7 cells overexpressing IRS-1, i.e., MCF-7/IRS-1, clone 3, exhibited increased transformation in the presence of serum or IGF-I [32], typical for these cells.

SHC associates with $\alpha5\beta1$ integrin (FN receptor) in MCF-7 cells, and the abundance of SHC/$\alpha5\beta1$ integrin complexes is increased in MCF-7/SHC cells. The limited or absent effects of SHC overexpression on mitogenic and transforming potential of MCF-7 cells prompted us to assess the role of SHC in nongrowth processes, specifically, adhesion and motility. Because interactions of cells with FN have been implicated in the growth and metastatic behavior of breast cancer cells [30, 31] and since SHC is a potential mediator of FN receptor signaling [18, 21], we investigated how overexpressed SHC affects the function of $\alpha5\beta1$ integrin in MCF-7 cells. The first observation was that the levels of SHC, especially $p46^{SHC}$, associated with $\alpha5\beta1$ were markedly increased (~sevenfold) in MCF-7/SHC clones compared with that in MCF-7 cells or in several cell lines with normal levels of SHC but overexpressing other signaling proteins (IRS-1 or the IGF-IR) (Fig. 2). Interestingly, the amount of $p46^{SHC}$ associated with $\alpha5\beta1$ integrin was similar in both MCF-7/SHC clones, regardless of the level of SHC overexpression (Fig. 2). This suggests that the extent of SHC/$\alpha5\beta1$ binding is not directly proportional to total cellular SHC levels and that $\alpha5\beta1$/SHC complex formation is a saturable process, possibly determined by the limited expression of $\alpha5\beta1$ integrin in MCF-7 cells [30].

MCF-7/SHC cells exhibit increased adhesion to FN. Because of the enhanced association of SHC with $\alpha5\beta1$ integrin in MCF-7/SHC cells, we examined the role of SHC in cellular interactions with FN using cell lines with normal, amplified, or reduced SHC levels. We found that the overexpression of SHC was associated with an accelerated cell adhesion to FN, while the reduction of SHC levels blocked cell spreading on the substrate (Fig. 3 and Table 2). The differences in the

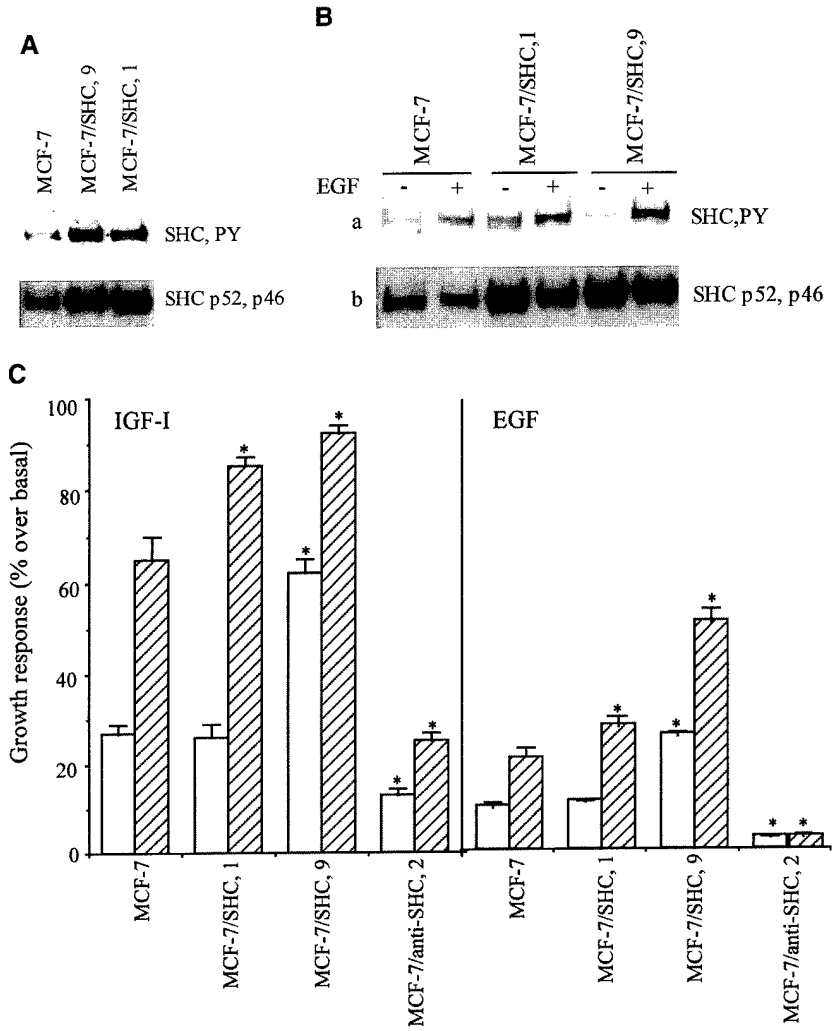

FIG. 1. MCF-7/SHC cells. (A) SHC expression and tyrosine phosphorylation in proliferating cells. The protein levels and tyrosine phosphorylation (PY) of $p52^{SHC}$ and $p46^{SHC}$ in two selected MCF-7/SHC clones 1 and 9 were determined in 750 μg of protein lysate by IP and WB with specific antibodies, as detailed under Materials and Methods. Cell lysates were isolated from logarithmic cultures maintained in normal growth medium. (B) SHC expression and tyrosine phosphorylation in growth-factor-stimulated cells. 70% confluent cultures were synchronized in PRF-SFM for 24 h and then stimulated with 10 ng EGF for 15 min. SHC levels and tyrosine phosphorylation (PY) were studied by IP and WB in 250 μg of protein lysates. Note that lane MCF-7/SHC, 1, EGF (−) is overloaded. (C) Growth response of MCF-7/SHC cells to IGF-I and EGF. The cells at 50% confluence were synchronized in PRF–SFM and stimulated with mitogens for 4 days as described under Materials and Methods. Abscissa, cell lines; ordinate, the percentage of growth increase over that in PRF-SFM. Solid bars, low doses: IGF-I 1 ng/ml or EGF 1 ng/ml; striped bars, high doses, IGF-I 20 ng/ml or EGF 10 ng/ml. High doses of IGF-I or EGF are the EC_{50} concentrations in these cells. SD is marked by solid bars; asterisks indicate statistically significant differences ($P \leq 0.05$) between the growth responses of MCF-7/SHC or MCF-7/anti-SHC cells and identically treated MCF-7 cells. The results are average of four experiments.

dynamics of cell interactions with FN were most evident at 1 h after plating (Fig. 3B and Table 2). Specifically, while at this time both MCF-7/SHC clones were well spread on FN, and almost no floating cells were observed, only ~50% of MCF-7 cells exhibited initial attachment to the substrate (cells were still rounded but with distinct membrane protrusions), and MCF-7/anti-SHC cells remained completely suspended. At 2 and 6 h after plating, MCF-7 and MCF-7/SHC clones were attached to FN and the differences in adhesion among these cell lines were unremarkable. At the same time, MCF-7/anti-SHC cells were in minimal contact with FN (Table 2). After 24 h, MCF-7/anti-SHC cells formed small aggregates demonstrating limited contact with the substrate, but all other tested cell lines (represented here by MCF-7 cells) were fully attached (Fig. 3D and Table 2). At 48 h, MCF-7/anti-SHC cells were completely detached, while MCF-7 and MCF-7/SHC cells begun proliferation on FN (data not shown).

Our experiments also indicated that the adhesion of MCF-7/SHC cells to FN was mediated by $\alpha 5\beta 1$ integrin, since this process was totally blocked with a spe-

TABLE 1

Anchorage-Independent Growth of MCF-7/SHC Cells

Cell line	Number of colonies				
	10% FBS	5% CS	SFM + IGF-I	SFM + EGF	SFM + IGF-I + EGF
MCF-7	172	105	2	1	12
MCF-7/SHC, 1	164	90	0	0	9
MCF-7/SHC, 9	155	88	0	0	10
MCF-7/IRS-1, 3	213	131	25	10	22

Note. The cells were tested in soft agar as described under Materials and Methods. The agar-solidified medium was either DMEM:F12 with 10% FBS or 5% CS or PRF–SFM with EGF (200 ng/ml), IGF-I (50 ng/ml), or EGF plus IGF-I (50 plus 200 ng/ml, respectively). MCF-7/IRS-1, clone 3, characterized by an increased transforming potential [30], was used as a positive control. The experiment was repeated seven times. Average number of colonies of the size at least 100 μm in diameter is given.

cific anti-$\alpha5\beta1$ blocking antibody (Fig. 3C), but not with a control goat IgG (not shown).

In contrast with the results obtained on FN, the dynamics of cell adhesion on COL, which is mediated by an integrin not associating with SHC ($\alpha5\beta1$) [18], were similar in all tested cell lines, regardless of the levels of SHC expression. Specifically, all cells tested initiated contacts with COL at 15 min and completed attachment at 1 h after plating (data not shown).

Overexpression of SHC modulates adhesion-dependent, but not growth factor-induced, MAP kinase activity on FN. Cell adhesion to ECM and the activation of different integrin-associated cytoplasmic TKs result in the stimulation of MAPK activity [35]. This process can be mediated through SHC, which, as a substrate of TKs (e.g., Fyn, other c-Src-like kinases, or FAK), is tyrosine phosphorylated, binds the GRB2/SOS complex, and stimulates Ras [18, 19, 21]. The integrin–MAPK pathway can also be induced in a SHC-independent way, through FAK–GRB2-SOS–Ras signaling [20, 21]. Here we studied the effect of SHC amplification on adhesion-dependent MAPK response in MCF-7 and MCF-7/SHC cells. Figure 4 demonstrates representative results obtained with MCF-7/SHC, 1 cells; the results with the clone MCF-7/SHC, 9 were similar.

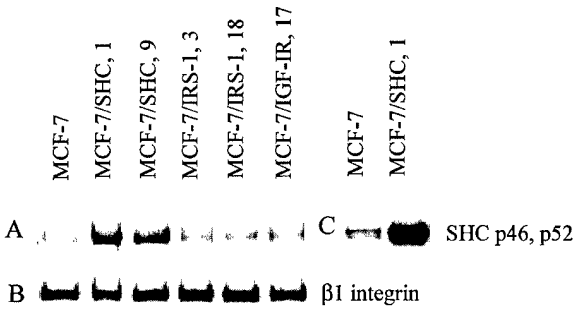

FIG. 2. SHC associates with $\alpha5\beta1$ integrin. The amounts of SHC associated with $\alpha5\beta1$ integrin in MCF-7/SHC cells, MCF-7 cells, and several control clones with normal SHC levels but increased levels of IRS-1 (MCF-7/IRS-1, clones 3 and 18) or the IGF-IR (MCF-7/IGF-IR, clone 17) were determined in 750 μg of protein lysate by IP with an anti-$\alpha5\beta1$ pAb and WB with an anti-SHC pAb (A). The expression of $\alpha5\beta1$ integrin in the cells was determined after stripping the above blot and reprobing with the anti-$\beta1$ pAb (only the β subunit is shown) (B). To locate the position of SHC isoforms on the gel, SHC proteins were precipitated from 250 μg of MCF-7 and MCF-7/SHC, 1 cell lysates with an anti-SHC pAb, run in parallel with $\alpha5\beta1$ integrin IP samples and probed with an anti-SHC mAb (C). Note: The $\alpha5\beta1$ integrin IP samples could not be reprobed with the SHC mAb because of strong antibody cross-reaction.

First, we found that overexpression of SHC significantly modulated MAPK activation in cells spread on FN, but on COL (Fig. 4A). Specifically, on COL, MCF-7 and MCF-7/SHC cells responded similarly—the activation of MAPK was biphasic, with a peak between 30 min and 4 h after plating, followed by a decline of activity at 8 h, and then an increased activity between 12 and 24 h. In contrast, on FN, the dynamics of MAPK response was different—in MCF-7 cells, the stimulation of MAPK was the highest at 1 h after plating and the kinases remained highly stimulated for up to 8 h. In MCF-7/SHC cells, MAPK was activated at 30 min after plating, reached the maximum at 1 h, and rapidly declined at 4 h to reach basal levels at 24 h (Fig. 4A). The activation of MAPK in suspended cells was undetectable (not shown).

Next, we investigated whether SHC overexpression affects growth-factor-induced MAPK response in cells plated on FN. We used EGF in this experiment since this mitogen induces SHC phosphorylation more strongly than IGF-I in MCF-7 cells (Surmacz *et al.*, unpublished observations). The patterns of EGF-stimulated MAPK activity were remarkably similar on COL and FN (a peak between 15 min and 1 h after treatment followed by a decline to basal levels) in both MCF-7 and MCF-7/SHC cells (Fig. 4B).

Since MAPK pathway contributes to cell growth, we studied whether the reduced duration of adhesion-dependent MAP activity reflects mitogenicity of MCF-7/

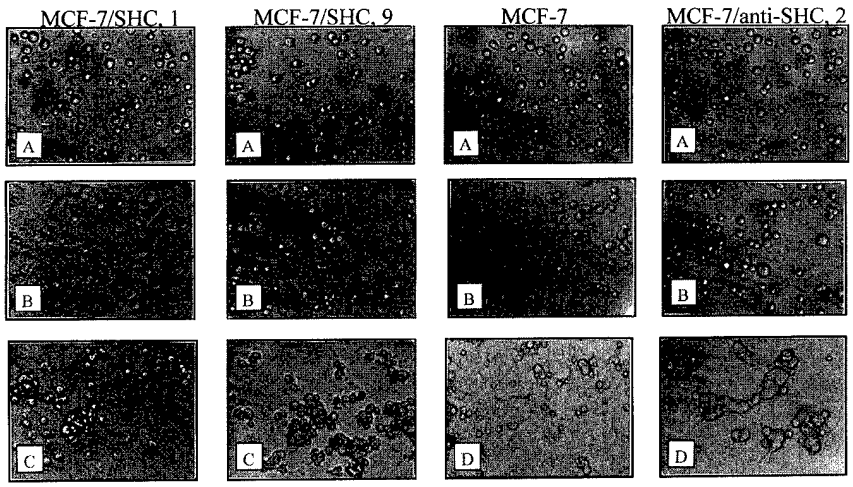

FIG. 3. Adhesion of MCF-7/SHC clones on FN. MCF-7/SHC clones 1 and 9 (amplified SHC), MCF-7 cells (normal SHC levels) and MCF-7/anti-SHC, clone 2 (reduced SHC levels) were synchronized for 24 h in PRF–SFM and plated on FN (50 μg/ml) in PRF–SFM. The cells were photographed at times 0 (A) and 1 h (B). The role of α5β1 integrin in the adhesion of MCF-7/SHC clones 1 and 9 was assessed by blocking the FN receptor with a specific antibody 30 min before cell plating (C), as described under Materials and Methods. The long-term (24 h) adhesion of MCF-7 and MCF-7/anti-SHC, clone 2, is shown in panels D.

SHC cells cultured on FN (Table 3). Indeed, we found that overexpression of SHC coincided with a significant (~twofold) growth inhibition. Interestingly, the addition of EGF (different doses, up to 100 ng/ml) to growth medium did not improve proliferation of MCF-7/SHC or MCF-7 cells on FN. The addition of IGF-I (doses up to 100 ng/ml) only minimally (9–22%) stimulated growth under the same conditions (Table 3).

Overexpression of SHC inhibits basal motility on FN, and IGF-I or EGF mobilizes MCF-7/SHC cells. We investigated whether increased binding of SHC to α5β1 integrin affects cell motility in FN-coated inserts. We found that basal migration of MCF-7/SHC cells was significantly (~fourfold) reduced compared with that of MCF-7 cells and several MCF-7-derived cell lines containing normal amounts of SHC (Fig. 5). In contrast, the motilities of MCF-7/SHC clones in COL-coated inserts were similar ($P \geq 0.05$) to those seen with other tested cell lines (Fig. 5).

The use of EGF or IGF-I as chemoattractants significantly (~five- to sevenfold, $P \leq 0.01$) improved the migration of MCF-7/SHC cells toward FN, but not to COL. The mitogens did not affect motility of other cells to COL, except some inhibition of MCF-7/IRS-1, clone 18 with 10 ng/ml EGF. Interestingly, in FN-coated inserts, 10 ng/ml EGF stimulated the migration of MCF-7/IRS-1 cells; however, the extent of this stimulation was much lower than that of SHC overexpressing clones (Fig. 5). The increased EGF sensitivity of MCF-7/IRS-1 clones has been noticed before [16].

TABLE 2

Dynamics of Cell Attachment to FN

	% Nonadherent cells					
Cell line	0 h	0.5 h	1 h	2 h	6 h	24 h
MCF-7	100	74	55	8	5	2
MCF-7/SHC, 1	100	33	5	5	4	4
MCF-7/SHC, 9	100	25	6	5	2	3
MCF-7/anti-SHC, 2	100	99	100	78	80	76

Note. 0.5×10^5 cells suspended in PRF–SFM were plated into 60-mm plates coated with 50 μg/ml FN as described under Materials and Methods. At the time of plating (0 h) and at 0.5, 1, 2, 6, and 24 h after plating, the floating cells were collected and counted. The values represent the percentages of cells floating vs cells originally plated and are averages from three experiments.

DISCUSSION

SHC is a signaling substrate of most receptor-type and cytoplasmic TKs and therefore may amplify various cellular responses [2]. In consequence, the significance of SHC amplification must depend on the intracellular and extracellular cell context. Breast cancer cells, unlike normal breast epithelium, frequently overexpress TKs, such as c-Src (80%), or ERB2 (~20–30%), which may result in constitutive activation of SHC [9, 10]. The contribution of amplified SHC signaling to development and progression of breast cancer is not known. We addressed this question by examining the effects of SHC overexpression in MCF-7 cells (representing an early stage of breast cancer and characterized by moderate c-Src amplification). The major findings of this work are that unlike in fibroblasts, hyperactivation of SHC is not sufficient to provide sig-

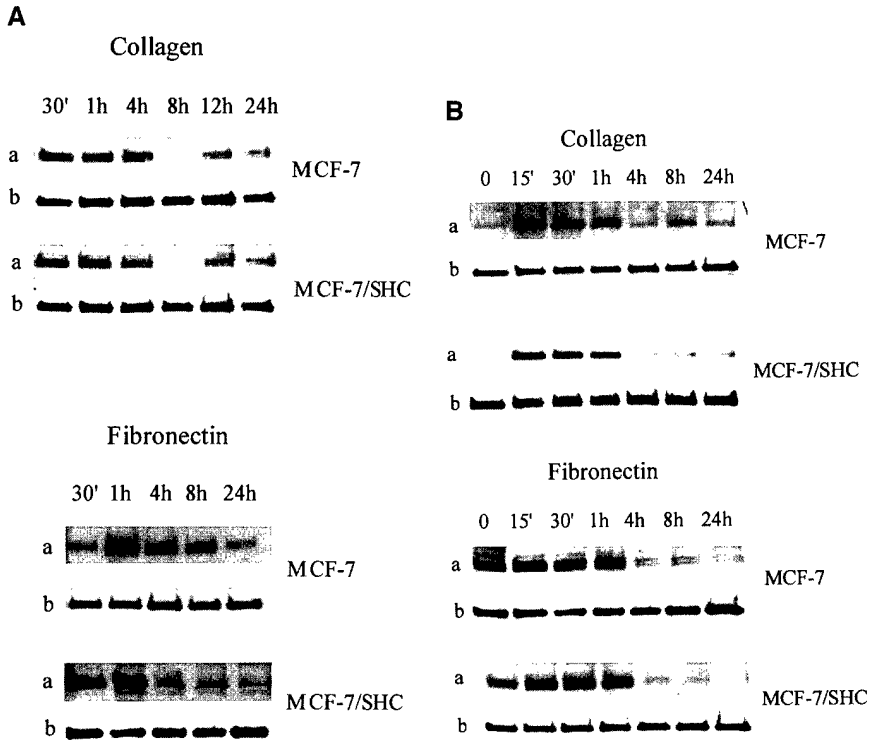

FIG. 4. Adhesion-induced (A) and EGF-dependent (B) MAPK activity in MCF-7/SHC cells. To measure adhesion-induced MAP kinase activity (A), MCF-7 and MCF-7/SHC cells were plated on COL IV or FN. The cells were lysed at the indicated times after plating. The phosphorylated forms of p42 and p44 MAPK were determined as described under Materials and Methods. EGF-induced MAP kinase activity (B) was determined in MCF-7 and MCF-7/SHC cells. The cells were plated on COL IV or FN, allowed to attach for 1 h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0–24 h) of the treatment. In (A) and (B), panels (a) represent phosphorylated MAPK, panels (b) total cellular levels of MAPK. The representative results demonstrating MAPK response in MCF-7 cells and MCF-7/SHC, clone 1, are shown; results with MCF-7/SHC, clone 9, were analogous to that obtained in clone 1.

nificant growth or transforming advantage in breast cancer cells. High levels of SHC, however, increase cell connections with FN and modulate cell growth and migration on this substrate, which may have consequences in cell spread and metastasis.

TABLE 3

Growth of MCF-7/SHC Cells on FN

	Cell number		
Cell line	5% CS	5% CS + EGF	5% CS + IGF-I
MCF-7	2.2×10^5	2.2×10^5	2.4×10^5
MCF-7/SHC, 1	1.1×10^5	1.0×10^5	1.3×10
MCF-7/SHC, 9	0.9×10^5	0.8×10^5	1.1×10^5

Note. The growth of cells on FN in normal growth medium (DMEM:F12 + 5% CS) or growth medium containing EGF (100 ng/ml) or IGF-I (100 ng/ml) was tested as described under Materials and Methods. The cells were plated at the concentration 0.5×10^5 cells/ml and counted after 4 days of culture. The values represent cell numbers/ml and are average results from three independent experiments.

SHC in epithelial cell growth and transformation. In mouse fibroblasts, overexpression of SHC resulted in increased SHC tyrosine phosphorylation, augmented EGF-, or IGF-I-dependent MAPK response, accelerated cell cycle progression through G1 phase in the absence of growth factors, and enhanced anchorage-independent growth in soft agar and tumorigenicity in nude mice [1, 6, 24]. Increased levels of SHC also potentiated growth factor response in myeloid and A549 adenocarcinama cells [3, 5]. Consistent with these findings are our previous data demonstrating that downregulation of SHC results in reduced sensitivity to mitogenic action of EGF and IGF-I and growth inhibition in breast cancer cells [16]. The present studies indicated that in SHC overexpressing epithelial cells, like in fibroblasts, basal and growth-factor-induced SHC tyrosine phosphorylation was increased, and cell responsiveness to IGF-I and EGF was moderately augmented in monolayer culture on plastic. However, amplification of SHC did not potentiate MAPK activity or proliferation of cells in complete serum-containing medium. This, again, was consistent with

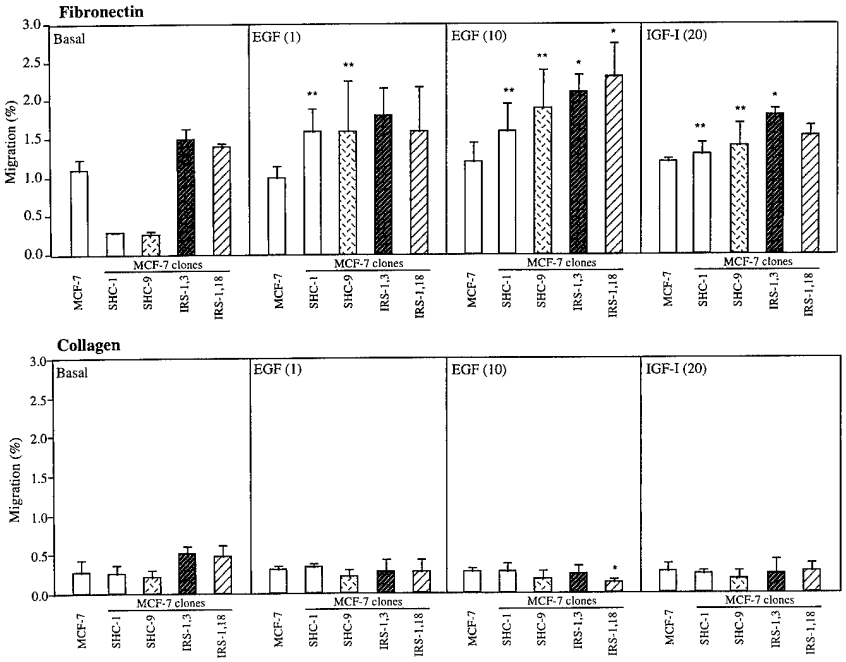

FIG. 5. Motility of MCF-7/SHC cells in FN or COL inserts. The motility of MCF-7/SHC cells and several control cell lines with normal SHC levels was tested as described under Materials and Methods. The upper and lower chambers contained PRF–SFM. Growth-factor-induced motility was assessed by supplementing PRF–SFM in lower chambers with either EGF (1 or 10 ng/ml) or IGF-I (20 ng/ml). The percentage of cells that migrated to the underside of inserts (relative to the number of cells plated) is shown. The experiments were repeated four times. Average values are given. Asterisks indicate statistically significant ($^*P \leq 0.05$, $^{**}P \leq 0.01$) differences between the basal and growth factor induced migration of a given cell line.

the effects observed in SHC overexpressing NIH 3T3 fibroblasts [6].

Note that high levels of SHC did not promote transformation of MCF-7 cells, whereas overexpression (at a similar level) of another signaling substrate IRS-1 markedly augmented anchorage-independent growth [32]. Since anchorage-independent growth reflects tumorigenic potential of breast cancer cells [36] and other cell types [1], our results indicate that, unlike in NIH 3T3 cells, SHC is not oncogenic in MCF-7 cells. This may reflect differences between pathways controlling transformation in fibroblasts and epithelial cells.

SHC in cell adhesion and motility. In contrast with the minimal impact of SHC overexpression on growth and transforming processes, high levels of SHC significantly modulated cell interactions with ECM in breast epithelial cells. SHC was found associated with $\alpha5\beta1$ integrin, the FN receptor, and $\alpha5\beta1$/SHC complexes were more abundant in SHC overexpressing cells than in other cell lines with SHC normal levels. The increased SHC/$\alpha5\beta1$ binding in MCF-7/SHC cells was paralleled by accelerated cell attachment to FN, reduced basal motility, abbreviated adhesion-mediated MAPK activity, and inhibited proliferation on the substrate. These effects were absent on COL (whose receptor does not bind SHC in MCF-7 cells), which suggests a specific role of SHC-$\alpha5\beta1$ interactions in the above processes.

The association of SHC with certain classes of integrins has been noted in several other cell systems. In A431 cells and other cell lines, binding and tyrosine phosphorylation of SHC to $\beta1$ integrin was induced by cell contact with ECM or by integrin cross-linking with a specific antibody [18]. Similarly, an association of SHC with $\alpha6\beta4$ integrin was observed in attached, but not suspended, A431 cells [34]. In several cell types, ligation of SHC-binding integrins, but not other integrins, has been reported to enhance cell cycle progression [18]. In our cell system, however, the increased association of SHC with $\alpha5\beta1$ integrin and the enhanced attachment to FN coincided with growth inhibition. Consistent with these findings are the observations of Wang *et al.,* who reported that in MCF-7 cells, $\alpha5\beta1$ integrin overexpression and improved interactions of cells with FN resulted in reduced proliferation on the substrate and impaired tumorigenicity *in vivo* [30].

Cell growth and survival on ECM are reflected by enhanced MAPK activity [18, 19, 35]. This pathway is induced by various integrin-associate TKs (e.g., c-Src-like TKs or FAK) and often involves activation of the SHC–GRB2–Ras pathway [19–21]. We found that the

amplification of SHC corresponded to the reduced duration of adhesion-mediated MAPK response on FN but not on COL. Note that, in mouse fibroblasts, an early decline of MAPK activity coincided with growth inhibition, whereas a prolonged activity marked cell cycle progression [37]. Thus, the abbreviated MAPK response in MCF-7/SHC cells may reflect their significantly slower proliferation on FN. In our experiments, treatment of cells spread on FN with EGF induced MAPK but did not stimulate cell growth, which confirms that MAPK signaling is required but not sufficient for the proliferation of MCF-7 cells [38].

Reduced growth and better attachment to FN in MCF-7/SHC cells were also associated with significantly reduced basal migration. However, EGF or IGF-I induced motility of MCF-7/SHC clones more strongly than other control cell lines when tested in FN-coated inserts. Such an enhancement of growth-factor-induced migration in SHC overexpressing cell lines has been reported before [5]. The increased chemotaxis was probably mediated by pathways other than MAPK, since MAPK activities were similar in MCF-7 and MCF-7/SHC cells treated with EGF.

In summary, in MCF-7 cells, the impact of the amplified SHC on cell growth and transformation is not significant; however, SHC plays an important role in the regulation of cell adhesion and motility on FN through its interaction with $\alpha 5\beta 1$ integrin. The significance of SHC-mediated interactions with FN in breast cancer metastasis is not known and will be pursued in an animal model.

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The Activated Insulin-Like Growth Factor I Receptor Induces Depolarization in Breast Epithelial Cells Characterized by Actin Filament Disassembly and Tyrosine Dephosphorylation of FAK, Cas, and Paxillin

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Insulin-like growth factor I (IGF-I) promotes the motility of different cell types. We investigated the role of IGF-I receptor (IGF-IR) signaling in locomotion of MCF-7 breast cancer epithelial cells overexpressing the wild-type IGF-IR (MCF-7/IGF-IR). Stimulation of MCF-7/IGF-IR cells with 50 ng/ml IGF-I induced disruption of the polarized cell monolayer followed by morphological transition toward a mesenchymal phenotype. Immunofluorescence staining of the cells with rhodamine–phalloidin revealed rapid disassembly of actin fibers and development of a cortical actin meshwork. Activation of phosphatidylinositol (PI)3-kinase downstream of the IGF-IR was necessary for this process, as blocking PI 3-kinase activity with the specific inhibitor LY 294002 at 10 μM prevented disruption of the filamentous actin. In parallel, IGF-IR activation induced rapid and transient tyrosine dephosphorylation of focal adhesion proteins p125 focal adhesion kinase (FAK), p130 Crk-associated substrate (Cas), and paxillin. This process required phosphotyrosine phosphatase (PTP) activity, since pretreatment of the cells with 5 μM phenylarsine oxide (PAO), an inhibitor of PTPs, rescued FAK and its associated proteins Cas and paxillin from IGF-I-induced dephosphorylation. In addition, PAO-pretreated cells were refractory to IGF-I-induced morphological transition. Thus, our findings reveal a new function of the IGF-IR, the ability to depolarize epithelial cells. In MCF-7 cells, mechanisms of IGF-IR-mediated cell depolarization involve PI 3-kinase signaling and putative PTP activities.

Key Words: **insulin-like growth factor I receptor signaling; F-actin; phosphatidylinositol 3-kinase; focal adhesion; phosphotyrosine phosphatase.**

INTRODUCTION

Differentiated epithelial cells form tightly adherent polarized sheets with a full complement of specific adhesive junctions that stably link cells together and to the underlying biological substratum [1]. In the cell interior, various membrane junctions are connected by the cytoskeletal network, providing the strength and architecture required for the proper physiological function of the epithelial sheet [2]. Normal epithelial cells move as a coherent sheet in which each cell keeps contacts with its neighboring cells as well as with extracellular matrix [3]. The ability of cells to move individually appears to be an exclusive attribute of carcinoma cells. At present, however, little is known about how depolarization of epithelial cells is accomplished and regulated and what signals trigger this process.

The studies on chemotaxis and cell spreading suggest that insulin-like growth factor I (IGF-I) is a regulator of motility in normal and tumor cells [4–7]. In breast cancer cells, IGF-I-induced chemotactic migration has been reported to occur through the IGF-I receptor (IGF-IR) [8, 9]. Importantly, IGF-IR expression is 14 times higher and IGF-IR kinase activity is significantly increased in malignant compared with normal breast tissue. Furthermore, ligands of the IGF-IR, IGF-I, and IGF-II, are secreted by the surrounding mammary epithelium stromal cells [10]. The possibility that IGF-IR overexpression promotes depolarization and locomotive functions in breast epithelial cells has not been investigated.

The IGF-IR belongs to the tyrosine kinase receptor superfamily and is known to play an important role in normal and abnormal growth [11]. Ligand binding stimulates autophosphorylation of the IGF-IR β subunit, elevates its kinase activity, and leads to a recruitment of multiple signaling molecules: insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), Shc, Crk, Gab1, Grb10, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) [12]. Many of the known IGF-IR pathways convey mitogenic stimuli. IRS-1 molecule, for example, via its multiple tyrosine phosphorylation sites, recruits secondary signaling proteins containing Src-homology 2 (SH2) domains such as PI

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3-kinase, phosphotyrosine phosphatase 1D (PTP1D/SH-PTP2/Syp), and Src-family kinase Fyn, as well as adapter proteins Grb-2, Crk, and Nck that participate in the mitogenic Ras/Raf/MAPK (mitogen-activated protein kinase) cascade [13]. It still remains unknown whether IGF-IR signaling pathways controlling epithelial cell motility are similar to or different from those required for cell proliferation.

The structure of the epithelial cell monolayer is supported by the integrity of the actin-enriched cytoskeleton, and its reorganization is critical for regulating cell motility [14]. Previous studies have described two types of IGF-IR effects on the actin cytoskeleton: the appearance of actin-enriched circular ruffles along the margins of KB epidermoid carcinoma cells [15, 16], and the development of an actin meshwork in the extended neurites of neuronal cells [17]. In breast epithelial cells, the relationship between the IGF-IR and the actin cytoskeleton has not been defined.

In polarized epithelial cells, focal adhesion proteins are localized to the termini of the stress fiber-like actin filaments as well as to cell–cell junctions adjacent to circumferential actin bundles [18]. Hence, reorganization of the actin network may affect the dynamics of focal adhesion assembly and lead to modulation of cell–substratum interaction, cellular shape, and consequently cell motility. In different cell types, IGF-I has been shown to positively or negatively modulate tyrosine phosphorylation of focal adhesion proteins such as FAK, Cas, and paxillin [17, 19, 20]. However, in these studies the link between IGF-I modulation of focal adhesion protein phosphorylation and cell motility was not established. Whether IGF-IR signaling regulates activity of focal adhesion proteins in breast epithelial cells, what the mechanism of the regulation might be, and how it relates to cell motility remain unexplored.

In work presented here, we analyzed the effect of IGF-IR activation on locomotion of MCF-7-derived breast epithelial cells organized in a monolayer with particular focus on elucidation of IGF-IR signaling promoting cell motility.

MATERIALS AND METHODS

Cell culture and chemicals. MCF-7 human breast epithelial cells overexpressing the IGF-IR were derived by stable transfection with pcDNA3/IGF-IR plasmid [21]. All MCF-7-derived cells were grown in DMEM:F12 (1:1) containing 5% calf serum. To starve the cells of serum, they were incubated in phenol red-free, serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.5 mg/ml bovine serum albumin (BSA), 1 μM $FeSO_4$, and 2 mM L-glutamine (SFM) for 24 h.

BSA, phenylarsine oxide (PAO), cytochalasin D (CD), and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin were purchased from Sigma. LY 294002 was from Calbiochem. Human recombinant IGF-I was purchased from Bachem.

Immunofluorescence light microscopy. Subconfluent cells grown on a glass coverslip were fixed with 3.7% formaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. To visualize actin filaments (F-actin) cells were stained with TRITC-conjugated phalloidin (1 μg/ml) for 30 min and examined with a Zeiss Axiophot microscope. Changes in the intracellular distribution of FAK, Cas, and paxillin were assessed by labeling with the primary antibodies: anti-FAK (A-17) pAb (Santa Cruz Biotechnology), a mixture of anti-Cas B and F pAbs (gift of Dr. A. H. Bouton, University of Virginia), or anti-paxillin mAb (Transduction Laboratories) for 60 min. In some experiments, subcellular localization of FAK was visualized with the 2A7 mAb raised against C terminus of pp125 FAK (gift of Dr. J. T. Parsons, University of Virginia). Primary antibody detection was performed with lissamine rhodamine (LRSC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Calbiochem). In controls, the primary antibody was omitted. Some samples were examined using the Zeiss Axiovert 100 MRC 600 confocal laser scanning (Bio-Rad) immunofluorescence microscope. Optical sections were taken at the ventral plane.

Immunoprecipitation and immunoblotting. Cells were lysed in protein lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 20 μg/ml aprotinin, 2 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride). FAK, Cas, and paxillin were precipitated from the cell lysates (500 μg of total protein) with specific antibodies: anti-FAK (A-17) polyclonal antibody (pAb) (Santa Cruz Biotechnology), anti-Cas monoclonal antibody mAb (Transduction Laboratories), and anti-paxillin mAb (Transduction Laboratories), respectively. Protein–antibody complexes were collected with either protein A– or anti-mouse IgG–agarose beads overnight. The precipitates were washed with HNTG buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Tyrosine phosphorylation and protein levels of FAK, Cas, and paxillin were assessed by immunoblotting with anti-phosphotyrosine mAb (PY-20) (Santa Cruz Biotechnology) or the specific antibodies as used for precipitations. The proteins were visualized by enhanced chemiluminescence detection (Amersham).

RESULTS

Activation of the IGF-IR Induces Depolarization of MCF-7 Cells

Serum-starved MCF-7 cells expressing moderate levels of the endogenous IGF-IR (6×10^4 receptors/cell) and MCF-7/IGF-IR cells with an 18-fold overexpression of wild-type IGF-IR both displayed a characteristic epithelial morphology with apicobasal polarity. The basal cell surfaces were adherent to the substratum, while lateral membranes of adjacent cells were attached to each other (Figs. 1A, 1B). In response to addition of 5–50 ng/ml IGF-I the cell monolayers underwent a dose-related morphological reorganization (data not shown). The most drastic changes occurred in 50 ng/ml IGF-I and were characterized by disruption of the epithelial sheet, loss of cell polarity, and development of a fibroblast-like phenotype by the majority of cells (Figs. 1C, 1D). Within 15 min cell–cell contacts loosened. By 60 min the cells partially detached from the plastic surface and rounded up slightly. Between 1 and 4 h of continuous IGF-I exposure the cells devel-

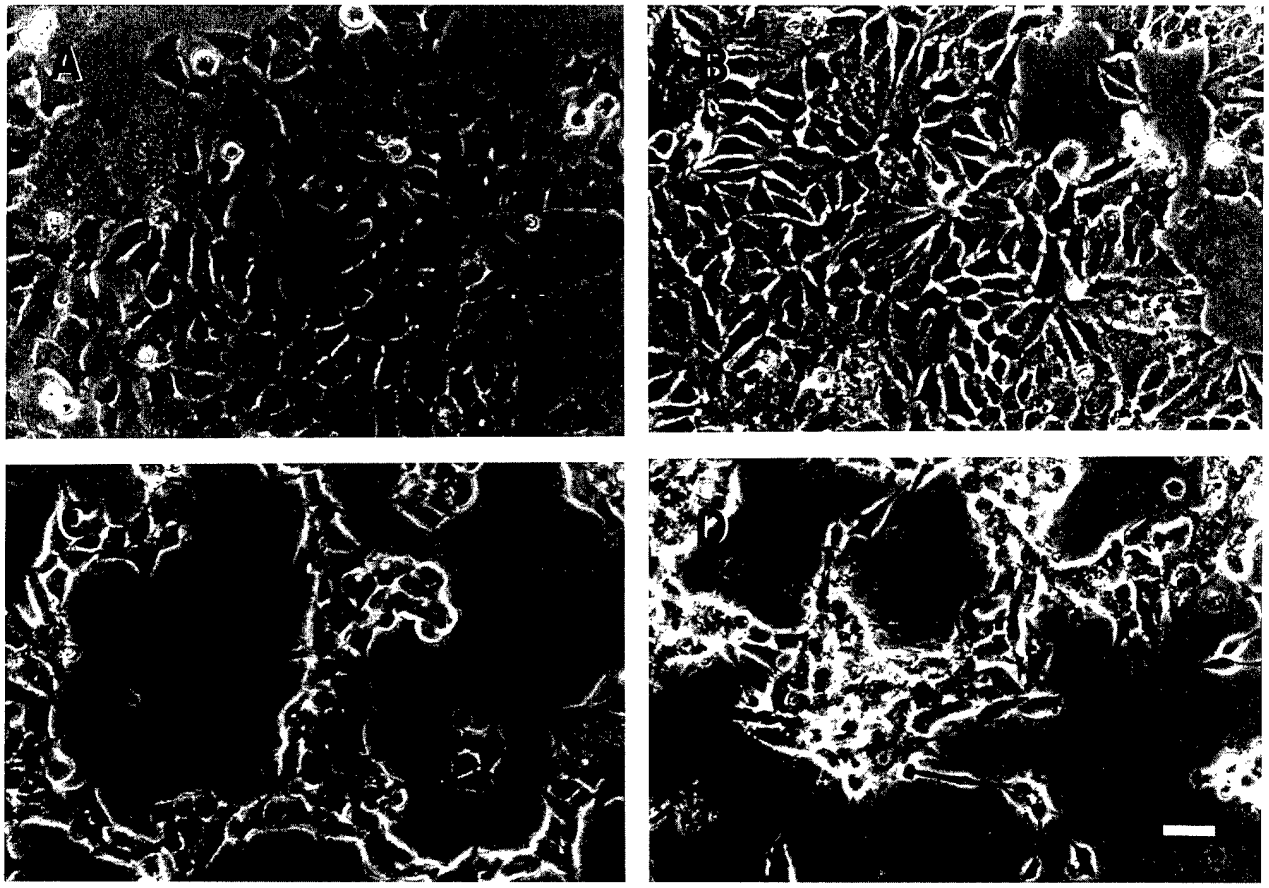

FIG. 1. IGF-IR activation stimulates depolarization and morphological transition in MCF-7-derived cells. The representative phase-contrast micrographs show the morphology of serum-starved MCF-7 (A) and MCF-7/IGF-IR (B) cells. In (C), MCF-7 and in (D), MCF-7/IGF-IR serum-starved cells were stimulated with 50 ng/ml IGF-I for 4 h. Bar = 100 μm.

oped multiple lamellipodial structures, which are characteristics of motile cells. Both control and IGF-IR-overexpressing cells were affected by IGF-I; however, the extent of the modifications was more pronounced in MCF-7/IGF-IR than MCF-7 cells (compare Figs. 1C and 1D). Therefore, IGF-IR-overexpressing cells were chosen for analysis in all subsequent experiments.

IGF-IR Activation Induces PI 3-Kinase-Dependent Disassembly of the Actin Filaments

To determine if IGF-IR activation induces reorganization of the actin cytoskeleton, F-actin was visualized in serum-starved MCF-7/IGF-IR cells treated with 50 ng/ml IGF-I for various times (Figs. 2A–2D). Within 5 min, the IGF-I effect on the actin cytoskeleton was detected as a disappearance of circumferential actin bundles, disassembly of stress fiber-like filaments, and development of a widespread cortical actin meshwork (Fig. 2B). After approximately 15 min, accumulation of F-actin was observed at the cell margins within structures resembling microspikes or small membrane ruffles (Figs. 2C, 2E). Between 1 and 4 h, the cells developed multiple extended protrusions, and fine long actin filaments reappeared, traversing cytoplasm and terminating in the veil-like lamellipodia (Fig. 2D).

Activation of PI 3-kinase has been implicated in restructuring of the actin cytoskeleton in other cell types [18, 26]. We investigated the role of PI 3-kinase in IGF-IR-induced modification of F-actin using a synthetic compound LY 294002 that blocks PI 3-kinase specifically (IC_{50} = 1.4 μM) and does not affect the activity of either IGF-IR or IRS-1 at concentrations up to 20 μM (data not shown). When MCF-7/IGF-IR cells pretreated with 10 μM LY 294002 for 30 min were stimulated with 50 ng/ml IGF-I for 30 min in the presence of the inhibitor, F-actin disassembly was prevented completely (compare Figs. 2E and 2F). The appearance of short actin bundles in membrane ruffles also was blocked. The actin cytoskeleton remained intact even 1 h after addition of IGF-I (data not shown).

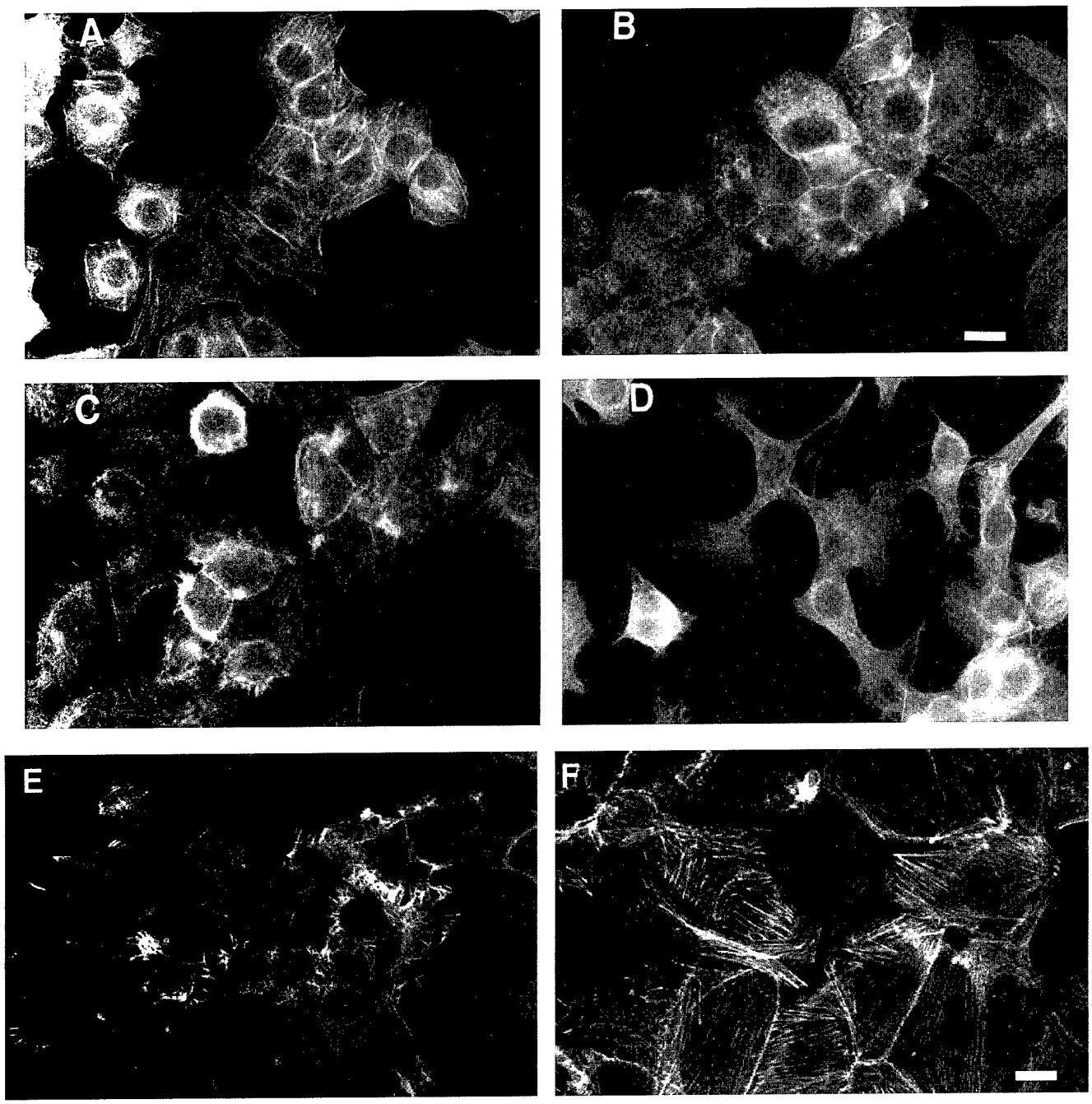

FIG. 2. IGF-I stimulation rapidly changes F-actin pattern in MCF-7/IGF-IR cells. The representative images of the selected time points show MCF-7/IGF-IR cells in which F-actin was visualized with TRITC-labeled phalloidin. Serum-starved cells (A); serum-starved cells stimulated with 50 ng/ml IGF-I for 5 min (B), 15 min (C), 30 min (E), and 4 h (D). (F) The cells were pretreated with 10 μM LY 294002 for 30 min, followed by stimulation with 50 ng/ml IGF-I for 30 min. Bar = 20 μm.

These results indicate that IGF-IR-activated PI 3-kinase signaling is critical for the initial step of IGF-IR-induced F-actin reorganization, namely, for the transient breakdown of actin filaments, as well as for subsequent F-actin rearrangement related to membrane protrusion.

The Activated IGF-IR Promotes Restructuring of Focal Adhesion Contacts

To investigate the relationship between changes in the actin filament network and organization of focal adhesions, conventional immunofluorescence light mi-

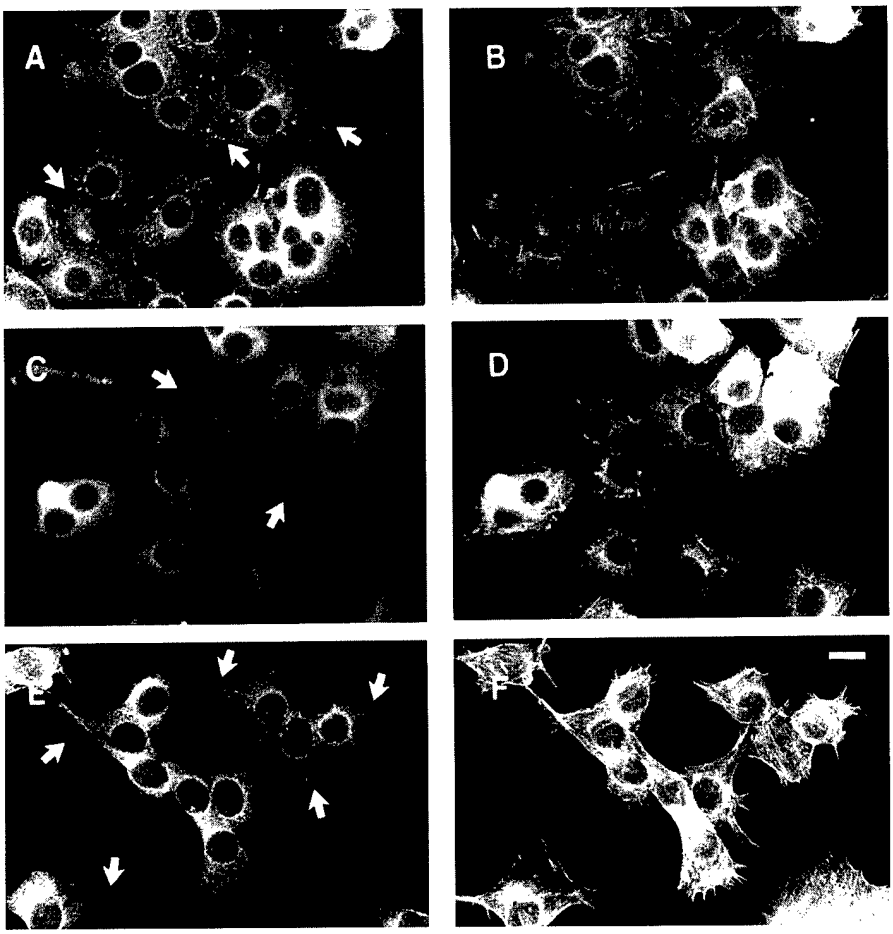

FIG. 3. Actin cytoskeleton reorganization is accompanied by redistribution of paxillin in IGF-I-stimulated MCF-7/IGF-IR cells. The representative images show cells costained with an anti-paxillin mAb (A, C, E) and TRITC–phalloidin (B, D, F). Serum-starved cells (A, B). Serum-starved cells treated with 50 ng/ml IGF-I for 5 min (C, D) and 60 min (E, F). Arrows in (A), (C), and (E) point to the position of the paxillin accumulations in membrane protrusions. Bar = 20 μm.

croscopy was used. MCF-7/IGF-IR cells stimulated with 50 ng/ml IGF-I for various times were examined (Figs. 3A–3F). Costaining of F-actin and paxillin, a marker of focal adhesions, revealed that "arrowhead"-shaped paxillin clusters disappeared, along with actin filament disassembly, within 5 min of IGF-I stimulation (compare Figs. 3A, 3B and 3C, 3D). Approximately 1 h after IGF-I addition, when the fine actin filaments reappeared, elongated streaks of paxillin staining were observed in numerous membrane protrusions (Figs. 3E, 3F).

To investigate whether changes in paxillin localization paralleled redistribution of two other focal adhesion-associated proteins, FAK and Cas, MCF-7/IGF-IR cells were doubly stained with anti-paxillin mAb [Figs. 4A(a)–4F(a)] and either anti-FAK (A-17) [Figs. 4A(b), 4C(b), 4E(b)] or anti-Cas [Figs. 4B(b), 4D(b), 4F(b)] pAbs. In fully spread, serum-starved cells, FAK and Cas partially colocalized with paxillin in punctate arrays of dots along cell edges and in fine clusters distributed over the ventral surface of cells (Figs. 4A, 4B). Within 15 min of IGF-I stimulation, FAK, Cas, and paxillin were all stained in the cytoplasm. At this time point the cells were slightly rounded up and had only a few paxillin-positive focal contacts (Figs. 4C, 4D). After 1–4 h, FAK and Cas could not be detected clearly in membrane protrusions where paxillin streaks were abundant [Figs. 4E(b), 4F(b)]. Similar results with respect to the intracellular redistribution of FAK were observed with both the A-17 and 2A7 anti-FAK antibodies.

Activation of the IGF-IR Induces Rapid Tyrosine Dephosphorylation of Focal Adhesion Proteins: FAK, Cas, and Paxillin

Actin fiber disassembly was shown to correlate with a reduction of FAK tyrosine phosphorylation in CHO cells [22]. We examined the time course and extent of the FAK tyrosine phosphorylation in MCF-7/IGF-IR

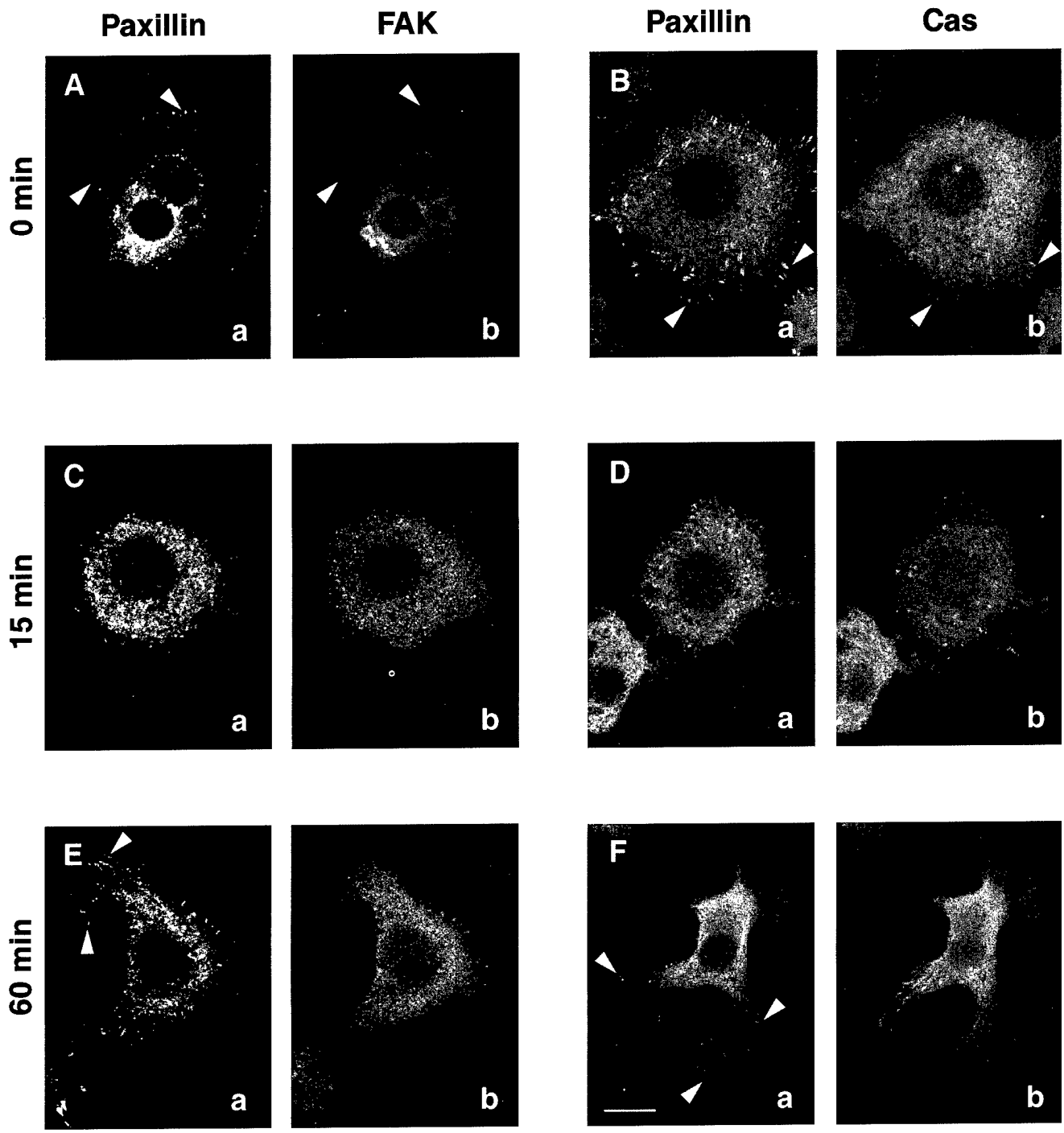

FIG. 4. IGF-I stimulation changes intracellular localization of FAK and Cas along with paxillin in MCF-7/IGF-IR cells. The representative confocal microscopy images demonstrate cells colabeled with anti-paxillin mAb [A(a), C(a), E(a)] and anti-FAK (N-17) pAb [A(b), C(b), E(b)]; cells colabeled with anti-paxillin mAb [B(a), D(a), F(a)] and a mixture of anti-Cas B and anti-Cas F pAbs [B(b), D(b), F(b)]. Serum-starved cells (A, B); serum-starved cells treated with 50 ng/ml IGF-I for 15 min (C, D) and 60 min (E, F). The representative areas of coincidental staining of paxillin with either FAK or Cas are marked by arrowheads in (A) and (B), respectively. In (E) and (F), arrowheads indicate the accumulation of paxillin in prolonged streaks localized to membrane protrusions. Bar = 20 μm.

cells stimulated with IGF-I. In serum-starved cells, FAK was prominently tyrosine phosphorylated (Fig. 5A′). Within 5 min of IGF-I treatment, FAK became dephosphorylated by 50% (Fig. 5A). The level of FAK phosphorylation remained low for at least 15 min, then was elevated almost to basal level by 1 h (Figs. 5A and

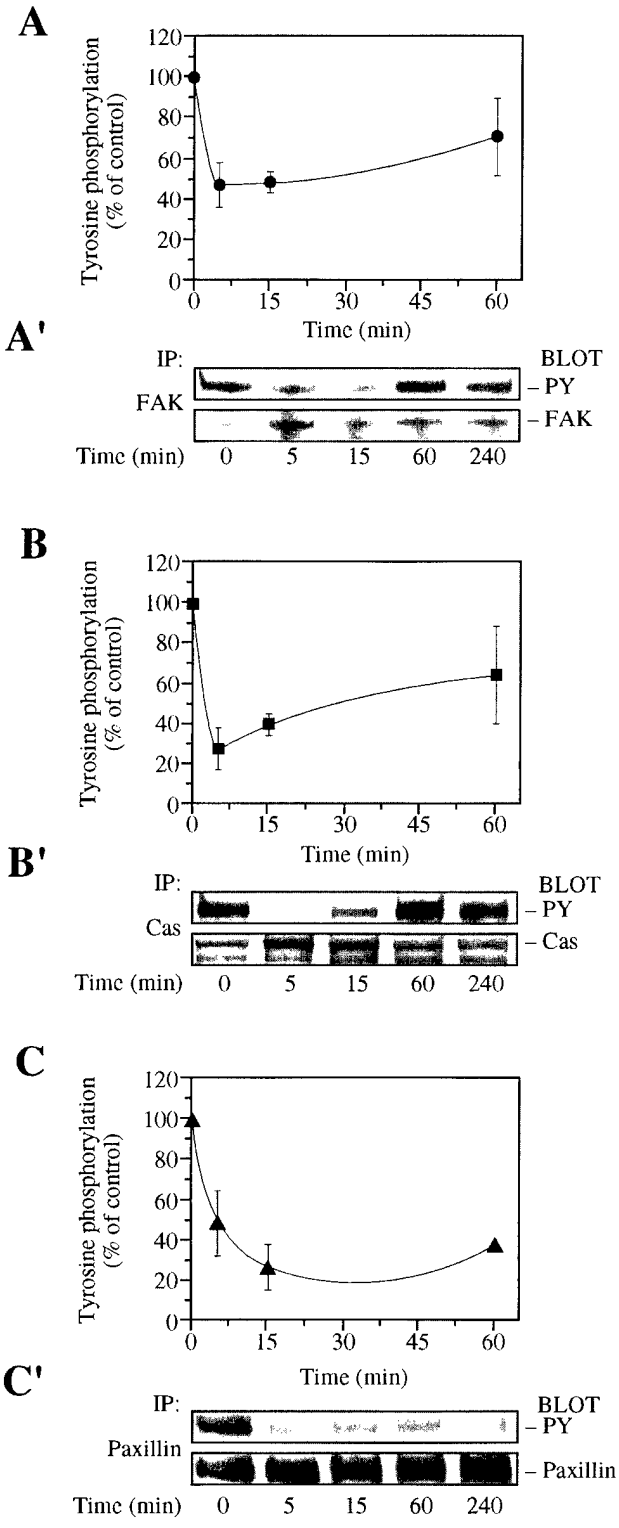

FIG. 5. IGF-IR activation induces rapid tyrosine dephosphorylation of FAK, Cas, and paxillin in MCF-7/IGF-IR cells. (A, B, C) Time courses of the relative tyrosine phosphorylation of FAK, Cas, and paxillin in response to 50 ng/ml IGF-I, correspondingly. The intensity of the bands of the phosphorylated proteins was measured by laser scanning densitometry. The value of tyrosine phosphorylation for each protein in serum-starved cells (time point 0 min) was taken as 100%. Error bars show SEM, $n = 4$. (A', B', C') Representative blots demonstrate the various levels of the tyrosine phosphorylated FAK, Cas, and paxillin (upper panels) and the comparable amounts of these proteins in the correspondent samples (lower panels) before and after IGF-I stimulation at the indicated time points. In the lower panels of (A'), (B'), and (C'), the anti-phosphotyrosine blots were stripped and reprobed with the indicated antibodies.

5A', upper panel). To test the possibility that tyrosine dephosphorylation of FAK affected the phosphotyrosine content of FAK targets such as Cas and paxillin, we immunoprecipitated both these proteins from the same lysates as FAK.

In control serum-starved cells, the tyrosine phosphorylation level of Cas and paxillin was high (Figs. 5B', 5C', upper panels). After 5 min of IGF-I stimulation, the Cas tyrosine phosphorylation was reduced by more than 70% of the control level. Tyrosine dephosphorylation of Cas was transient, since within 15 min of IGF-I addition its phosphorylation was elevated, and by 1 h it almost reverted to the basal level (Figs. 5B, 5B'). Within 5 min of IGF-I stimulation, tyrosine phosphorylation of paxillin was reduced by 50%, and over the next 10 min it declined further to 30–40% of basal phosphorylation, remaining at this level for at least 4 h (Fig. 5C).

In the absence of IGF-I stimulation, the basal level of tyrosine phosphorylation of FAK, Cas, and paxillin in the parental MCF-7 cells was comparable to that in MCF-7/IGF-IR cells. However, no significant changes in tyrosine phosphorylation status of focal adhesion-associated proteins FAK, Cas, and paxillin were observed in MCF-7 cells treated with 50 ng/ml IGF-I for up to 4 h (data not shown).

PAO Prevents Tyrosine Dephosphorylation of FAK, Cas, and Paxillin and Inhibits Membrane Protrusion in IGF-I-Stimulated MCF-7/IGF-IR Cells

To investigate whether protein tyrosine phosphatase activity was required for the reduced tyrosine phosphorylation of FAK, Cas, and paxillin in response to IGF-I stimulation of MCF-7/IGF-IR cells, we attempted to block a putative IGF-IR-activated PTP. The trivalent arsenical compound PAO was selected for this purpose. PAO is a membrane-permeable PTP inhibitor that blocks tyrosine phosphatase activity in insulin signaling and upregulates tyrosine phosphorylation of FAK and paxillin in fibroblasts at concentrations of 1–5 μM [23, 24].

In a series of control experiments we established that 5 μM PAO had no effect on IGF-I-induced tyrosine phosphorylation of either the IGF-IR β subunit or IRS-1 (data not shown). Subsequently, we examined

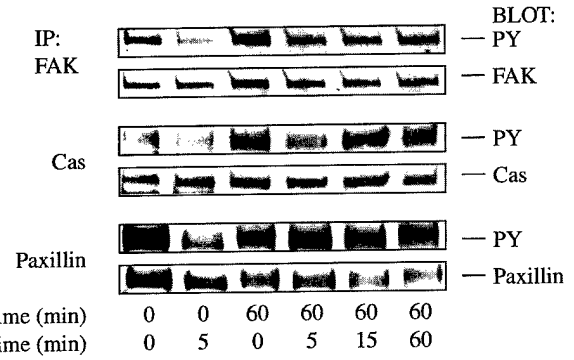

FIG. 6. PAO prevents tyrosine dephosphorylation of focal adhesion proteins in cells stimulated with IGF-I. Western blots show tyrosine phosphorylation and protein levels of the immunoprecipitated FAK, Cas, and paxillin in samples of serum-starved cells, serum-starved cells stimulated with 50 ng/ml IGF-I for 5 min, and serum-starved cells pretreated with 5 μM PAO for 60 min and then stimulated with 50 ng/ml IGF-I for the time indicated.

the effect of PAO on IGF-I-induced dephosphorylation of focal adhesion proteins FAK, Cas, and paxillin (Fig. 6). Preincubation with PAO for 60 min resulted in slightly increased basal tyrosine phosphorylation only of FAK and Cas. The following stimulation of PAO-treated cells with IGF-I for up to 60 min did not reduce tyrosine phosphorylation of FAK, Cas, and paxillin, indicating that PTP activity is required for IGF-IR-mediated dephosphorylation of these molecules.

We further investigated whether PAO rescue of the focal adhesion proteins from IGF-I-induced dephosphorylation influenced IGF-IR-mediated changes in cell morphology. MCF-7/IGF-IR cells were incubated in either 5 μM PAO in SFM or SFM alone for 60 min (Figs. 7A and 7D, respectively). Next, both cell monolayers were stimulated with 50 ng/ml IGF-I, and cell morphology was recorded after 15 and 60 min (Figs. 7B, E and 7C, F). After 60 min, PAO-treated cells formed lobopodium-like membrane advances resembling those displayed by serum-starved cells in response to a 15-min treatment with IGF-I (compare Figs. 7C and 7E). The outward extensions of lamellipodia typically seen in control cells after 60 min of IGF-I addition appeared to be blocked in PAO-pretreated cells, thereby suggesting at least a partial inhibition of morphological transition toward the motile phenotype.

DISCUSSION

The structural organization of a normal polarized epithelium restricts the separation and movement of individual cells. However, at early stages of malignant progression loss of epithelial polarity can promote cell motility and facilitate dissemination of cancer cells from epithelial tissue, thereby increasing the chance of a metastatic spread [25].

The increased level and enhanced autophosphorylation of the IGF-IR observed in malignant versus normal human breast tissues [10], as well as association of the IGF-IR with cell migration *in vitro* [8, 9], prompted our current investigations on the cellular and molecular mechanisms governing IGF-IR-mediated motility in breast cancer cells.

In the present work, we assessed the effects of IGF-IR overexpression on the events related to motility of MCF-7 breast epithelial cells. For the first time, we demonstrated that activation of the overexpressed IGF-IR causes depolarization of an epithelial cell monolayer. The magnitude of IGF-IR signaling as determined by the number of activated IGF-IRs appears critical for converting epithelial cells from a polarized to a motile phenotype, since a more striking morphological transformation occurred in MCF-7/IGF-IR (1.1×10^6 receptors/cell) than in the parental MCF-7 (6.0×10^4 receptors/cell) cells.

The drastic changes in morphology of IGF-I-stimulated MCF-7/IGF-IR cells point to the involvement of cytoskeleton restructuring in cell depolarization. In this study we analyzed reorganization of the actin cytoskeleton in MCF-7/IGF-IR cells stimulated with IGF-I for 4 h, and identified three distinct phases of F-actin reorganization: (1) actin filament disassembly followed by (2) accumulation of condensed short actin bundles along the cell periphery, and (3) reassembly of the long F-actin bundles.

We have demonstrated that disruption of circumferential and stress fiber-like actin bundles as well as cortical actin filaments—the cytoskeletal structures responsible for the architecture and strength of the multicellular epithelial sheet—is an early event associated with IGF-IR-induced depolarization. Disassembly of actin filamentous network appeared to be transient (5–15 min). How does the activated IGF-IR cause F-actin disassembly? One possibility is that the rapid breakdown of the actin filaments is caused by the activation of actin-severing proteins, like gelsolin, whose stimulation, in turn, requires the transient increase in intracellular Ca^{2+} [26]. In some cell types, IGF-I stimulates phosphatidylinositide turnover and intracellular Ca^{2+} release [27]. Whether a rise in Ca^{2+} and stimulation of the actin-severing proteins occur following IGF-IR activation in MCF-7 cells remains to be determined.

Another possibility might be an IGF-IR-mediated activation of the small GTP-binding protein Rac as well as other members of the Rho family. Recently, microinjection studies on Rho D, Rho G, TC10, Rnd 1, and Rnd 3 (RhoE) demonstrated that these proteins cause breakdown of the stress fibers and associated loss of cell adhesion in fibroblasts [28]. In mammary epithelial cells, Cdc42- and Rac-mediated actin reorganization has been disrupted by inhibition of the PI 3-kinase

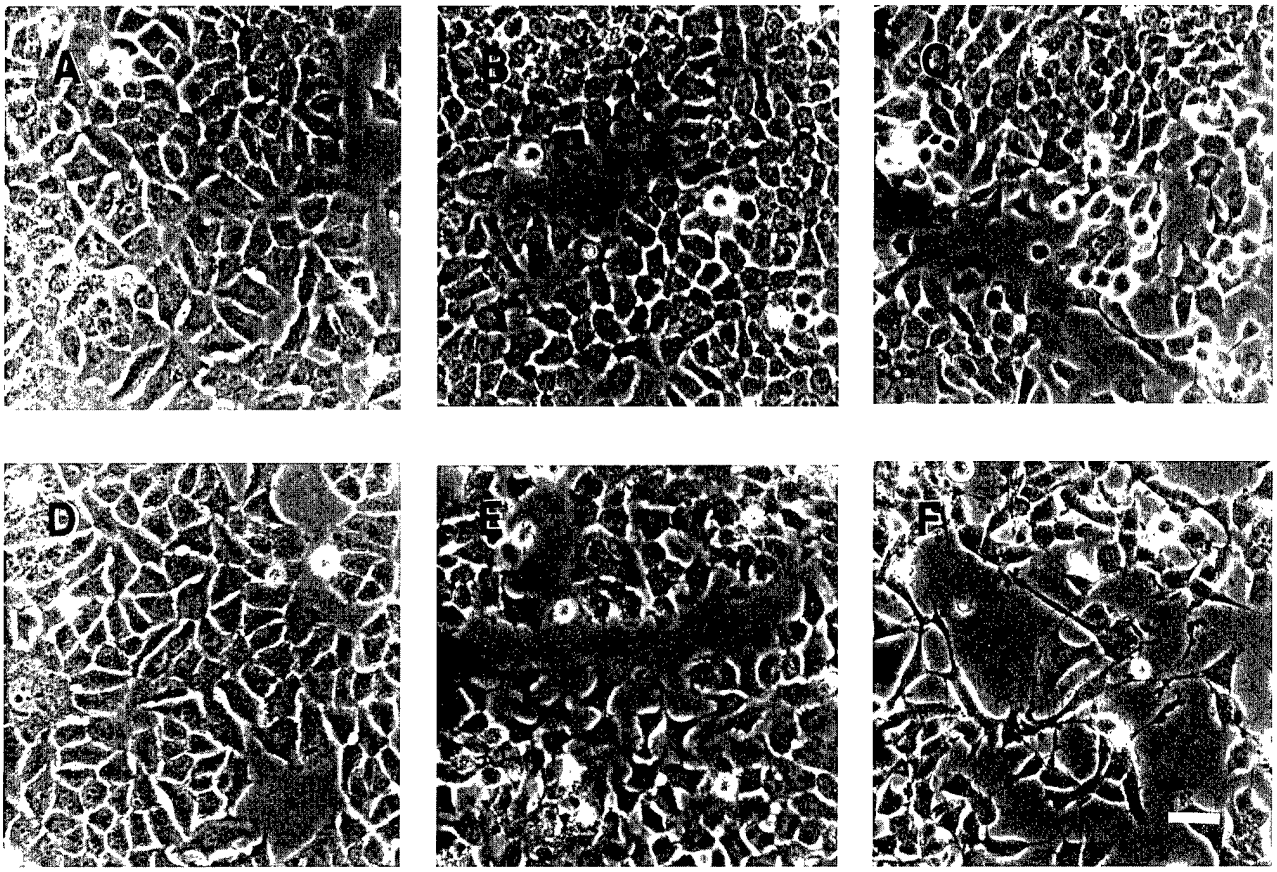

FIG. 7. PAO blocks morphological transitions in MCF-7/IGF-IR cells stimulated with IGF-I. The representative phase-contrast micrographs show the morphology of MCF-7/IGF-IR cells under different conditions. Serum-starved cells were pretreated with 5 μM PAO in SFM for 60 min (A) and then stimulated with 50 ng/ml IGF-I for either 15 min (B) or 60 min (C). In parallel, serum-starved cells (D) were stimulated with 50 ng/ml IGF-I for either 15 min (E) or 60 min (F). Bar = 100 μm.

[29]. In fibroblasts expressing the Ras oncogene, active PI 3-kinase has been implicated in the disruption of actin stress fibers via dissociation of cortactin, an F-actin crosslinking protein, from the actin–myosin II complex [30]. According to our previous data, the amount and activity of IRS-1-associated PI 3-kinase are higher in MCF-7/IGF-IR than in MCF-7 cells stimulated with IGF-I [21, 31]. In the study reported here, we tested the involvement of the PI 3-kinase in F-actin rearrangements in MCF-7/IGF-IR cells. Preincubation of the cells with the specific PI 3-kinase inhibitor LY 294002 abrogated IGF-I-induced F-actin disassembly, suggesting that IGF-IR/PI 3-kinase-dependent signaling triggers the rapid F-actin breakdown in MCF-7/IGF-IR cells.

The second phase of IGF-IR modulation of the actin cytoskeleton lasted approximately 1 h and was characterized by organization of the short F-actin bundles into microspike-like structures along the cell periphery. We speculate that IGF-IR regulation of actin-binding/bundling proteins is involved in microspike formation, which in turn might facilitate cell depolarization. This hypothesis is being tested currently.

The reappearance of the long actin filaments in the nascent lamellipodia apparently required actin polymerization, since addition of IGF-I along with cytochalasin, the drug that caps the plus end of actin filament and blocks actin polymerization, prevented lamellipodial extension (Guvakova, unpublished data). In many cell types, actin polymerization is closely linked with PI 3-kinase signaling [32], which also is required for lamellipodium formation and membrane ruffling [33]. Accordingly, we demonstrated that a block of PI 3-kinase activity inhibited IGF-I-induced membrane protrusion. This, however, does not rule out the possibility that the blockade of membrane extension is a secondary event, since the inhibition of PI 3-kinase also prevented F-actin disassembly, and the latter preceded lamellipodial protrusion in MCF-7/IGF-IR cells.

Previous studies have revealed that the breakdown of cell–cell adhesions plays an important role in epithelial–mesenchymal transition induced by different

growth factors [34, 35]. However, morphological cell transformation from polarized to nonpolarized phenotype is certainly related to modulation of cell–substratum interaction, and, therefore, mechanisms must exist to regulate the assembly and disassembly of cell–matrix junctions. We have shown here that IGF-IR activation induces rapid reorganization of focal adhesions. In response to IGF-I, "arrowhead"-shaped paxillin clusters in the static cell transformed into elongated streaks of paxillin at the periphery of membrane protrusions. The latter might function similarly to "transient focal contacts" of slowly moving cells described by Couchman and Rees [36]. Paxillin possesses multiple protein–protein interaction motifs [37] and, in contrast to FAK and Cas, is prominent in membrane protrusions. It is tempting to speculate that an exchange in paxillin molecular partners plays a key role in IGF-IR-mediated turnover of focal adhesions.

The effect of IGF-I on the tyrosine phosphorylation status of the focal adhesion proteins has been investigated in various cell models, and the reported influence of IGF-I on either FAK or paxillin appeared to be contrasting in different cells [17, 19, 20]. Recently, it was demonstrated that in IGF-I-stimulated Swiss 3T3 fibroblasts expressing very low levels of the IGF-IR there was an increase in FAK, Cas, and paxillin tyrosine phosphorylation [38]. In contrast to MCF-7 cells, however, serum-starved Swiss 3T3 cells lose their stress fibers and associated focal adhesions as well as tyrosine phosphorylation of FAK and paxillin [39]. Therefore, the contrasted cellular context and varying levels of IGF-IR expression in different cellular systems make it difficult to compare the results.

To clarify the relationship between IGF-IR stimulation, focal adhesion protein activity, and cell locomotion, we analyzed the time course of tyrosine phosphorylation of FAK, Cas, and paxillin in MCF-7 and MCF-7/IGF-IR cells exposed to IGF-I. In the MCF-7 parental cells, the relatively high basal phosphorylation of FAK, Cas, and paxillin was not reduced notably on IGF-I stimulation, whereas in MCF-7/IGF-IR cells, stronger IGF-IR activation triggered an acute tyrosine dephosphorylation of FAK and Cas. The kinetic profiles of FAK and Cas dephosphorylation were similar, consistent with the regulatory role of FAK in direct or indirect (via the activation of Src-family kinases) tyrosine phosphorylation of Cas [40, 41]. Alternatively, the activated IGF-IR might stimulate a PTP controlling the function of FAK, Cas, or Src-family proteins. In this respect it is interesting that a newly discovered dual-specificity phosphatase PTEN (MMAC1) interacts with FAK and reduces tyrosine phosphorylation of this kinase as well as Cas [42]. The SH3 domain of Cas also directly binds to proline-rich region of the cytoplasmic PTP1B [43] whose role in IGF-IR signaling remains obscure. In the present study, we found that another FAK-associated phosphoprotein, paxillin, underwent tyrosine dephosphorylation in response to IGF-I. The recent discovery of the physical association between a nonreceptor PTP–PEST and paxillin suggests that paxillin itself or paxillin-binding partners, including FAK, might serve as PTP–PEST substrates [44]. Another enticing candidate for a role in IGF-IR-regulated PTP activity is PTP1D (also known as Shp2/SH-PTP2/Syp). This cytoplasmic PTP is activated by direct binding to the phosphorylated insulin receptor (IR), IGF-IR or IRS-1 [45], and is known to mediate insulin-induced dephosphorylation of FAK and paxillin in CHO cells overexpressing the IR [46]. Recent studies identified an important role for PTP1D in regulating cell spreading, migration, and cytoskeletal architecture in fibroblasts, presumably via control of FAK [47].

Despite the unknown identity of IGF-IR-activated PTP in our cell model, the experiments with PAO, a phosphotyrosine phosphatase inhibitor, support the idea that induction of a PTP is critical for depolarization in MCF-7/IGF-IR cells. PAO rescue of FAK, Cas, and paxillin, from IGF-I-induced tyrosine dephosphorylation was sufficient to block development of the motile membrane protrusions. Collectively, our findings suggest the IGF-IR as an upstream regulator of a PAO-sensitive PTP, which either directly or indirectly dephosphorylates FAK, Cas, and paxillin and disrupts focal contacts.

In summary, we have established that activation of the overexpressed IGF-IR in MCF-7 cells results in a loss of epithelial cell polarity associated with acute disassembly of the actin filaments, subcellular reorganization of paxillin-enriched focal contacts, and rapid tyrosine dephosphorylation of FAK, Cas, and paxillin. Both PI 3-kinase and PTP activities are required for the depolarization of MCF-7 breast cancer cells. Taken together, these results suggest that IGF-IR overexpression in mammary epithelial cells may have a significant impact in stimulating tumor cell motility.

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INSULIN RECEPTOR SUBSTRATE 1 IS A TARGET FOR THE PURE ANTIESTROGEN ICI 182,780 IN BREAST CANCER CELLS

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The pure antiestrogen ICI 182,780 inhibits insulin-like growth factor (IGF)-dependent proliferation in hormone-responsive breast cancer cells. However, the interactions of ICI 182,780 with IGF-I receptor (IGF-IR) intracellular signaling have not been characterized. Here, we studied the effects of ICI 182,780 on IGF-IR signal transduction in MCF-7 breast cancer cells and in MCF-7-derived clones overexpressing either the IGF-IR or its 2 major substrates, insulin receptor substrate 1 (IRS-1) or src/collagen homology proteins (SHC). ICI 182,780 blocked the basal and IGF-I-induced growth in all studied cells in a dose-dependent manner; however, the clones with the greatest IRS-1 overexpression were clearly least sensitive to the drug. Pursuing ICI 182,780 interaction with IRS-1, we found that the antiestrogen reduced IRS-1 expression and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I. Moreover, in IRS-1-overexpressing cells, ICI 182,780 decreased IRS-1/p85 and IRS-1/GRB2 binding. The effects of ICI 182,780 on IGF-IR protein expression were not significant; however, the drug suppressed IGF-I-induced (but not basal) IGF-IR tyrosine phosphorylation. The expression and tyrosine phosphorylation of SHC as well as SHC/GRB binding were not influenced by ICI 182,780. In summary, downregulation of IRS-1 may represent one of the mechanisms by which ICI 182,780 inhibits the growth of breast cancer cells. Thus, overexpression of IRS-1 in breast tumors could contribute to the development of antiestrogen resistance. *Int. J. Cancer* 81:299–304, 1999.

ICI 182,780, an alpha-alkylsulfinylamide, is a new-generation pure antiestrogen (Wakeling *et al.,* 1991). The drug has shown great promise as a second-line endocrine therapy agent in patients with advanced breast cancer resistant to the non-steroidal antiestrogen tamoxifen (Tam). Indeed, in several *in vitro* and *in vivo* studies, the antitumor effects of ICI 182,780 were greater than those of Tam (Nicholson *et al.,* 1996; Osborne *et al.,* 1995; de Cupis *et al.,* 1995; Chander *et al.,* 1993; Wakeling *et al.,* 1991). Moreover, unlike Tam, ICI 182,780 lacks agonist (estrogenic) activity and its administration does not appear to be associated with deleterious side effects such as induction of endometrial cancer or retinopathy (Osborne *et al.,* 1995; Chander *et al.,* 1993). ICI 182,780 antagonizes multiple cellular effects of estrogens by impairing the dimerization of the estrogen receptor (ER) and by reducing ER half-life (de Cupis and Favoni, 1997; Chander *et al.,* 1993). ICI 182,780 also interferes with growth factor-induced growth, but it is not clear whether this activity is mediated exclusively through the ER, or if some ER-independent mechanism is implicated (de Cupis *et al.,* 1995). Despite their great antitumor effects, pure antiestrogens do not circumvent the development of antiestrogen resistance, as most breast tumor cells initially sensitive to ICI 182,780 eventually become unresponsive to the drug (de Cupis and Favoni, 1997; Pavlik *et al.,* 1996; Nicholson *et al.,* 1995). The mechanism of this resistance is not clear, but it has been suggested that both mutations of the ER as well as alterations in growth factor-dependent mitogenic pathways may be involved (de Cupis and Favoni, 1997; Larsen *et al.,* 1997; Pavlik *et al.,* 1996; Wiseman *et al.,* 1993).

The insulin-like growth factor (IGF) system [IGFs, IGF-I receptor (IGF-IR) and IGF binding proteins (IGFBP)] plays a critical role in the pathobiology of hormone-responsive breast cancer (reviewed in Surmacz *et al.,* 1998). In the experimental setting, IGF-IR has been shown to stimulate growth and transformation, improve survival, as well as regulate cell-cell and cell-substrate interactions in breast cancer cells (Surmacz *et al.,* 1998). Moreover, overexpression of different elements of the IGF system, such as IGF-II, IGF-IR or insulin receptor substrate 1 (IRS-1), provides breast cancer cells with growth advantage and reduces or abrogates estrogen growth requirements (Surmacz *et al.,* 1998). On the other hand, downregulation of IGF-IR expression, inhibition of IGF-IR signaling and reduced bioavailability of the IGFs have all been demonstrated to block proliferation and survival as well as to interfere with motility or intercellular adhesion in breast cancer cells (Surmacz *et al.,* 1998).

Clinical studies confirm the role of the IGF-I system in breast cancer development. First, IGF-IR has been found to be up to 14-fold overexpressed in breast cancer compared with its levels in normal mammary epithelium (Surmacz *et al.,* 1998; Resnik *et al.,* 1998; Turner *et al.,* 1997). Moreover, cellular levels of IGF-IR or its substrate IRS-1 correlate with cancer recurrence at the primary site (Rocha *et al.,* 1997; Turner *et al.,* 1997). The ligands of IGF-IR, IGF-I and IGF-II, are often present in the epithelial and/or stromal component of breast tumors, indicating that an autocrine or a paracrine IGF-IR loop may be operative and involved in the neoplastic process (Surmacz *et al.,* 1998). In addition, endocrine IGFs probably also contribute to breast tumorigenesis since the levels of circulating IGF-I correlate with breast cancer risk in premenopausal women (Hankinson *et al.,* 1998).

ICI 182,780 interferes with the IGF-I system in breast cancer cells. The antiestrogen has been shown to attenuate IGF-I-stimulated growth, modulate expression of IGFBPs and downregulate IGF binding sites (Surmacz *et al.,* 1998; de Cupis and Favoni, 1997; de Cupis *et al.,* 1995). The interactions of ICI 182,780 with the IGF-IR signaling pathways, however, have not been characterized.

Our previous work demonstrated that in breast cancer cells, Tam interferes with the IGF-IR signaling acting upon IGF-IR substrates IRS-1 and src/collagen homology proteins (SHC) (Guvakova and Surmacz, 1997). Normally, activation of IGF-IR results in the recruitment and tyrosine phosphorylation of IRS-1 and SHC, followed by their association with several downstream effector proteins and induction of various signaling pathways (Surmacz *et al.,* 1998). For instance, association of either IRS-1 or SHC with GRB-2/SOS complexes activates the Ras/MAP pathway, whereas binding of IRS-1 with p85 stimulates PI-3 kinase. Tam treatment blocks IGF-dependent growth, which coincides with decreased tyrosine phosphorylation of IRS-1 and IGF-IR and with hyperphos-

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phorylation of SHC (Guvakova and Surmacz, 1997). Here, we demonstrate the interactions of ICI 182,780 with IGF-IR signaling and discuss the relevant similarities and differences in the modes of action of the 2 antiestrogens.

MATERIAL AND METHODS

Cell lines and cell culture conditions

We used MCF-7 cells and several MCF-7-derived clones overexpressing either IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells) or SHC (MCF-7/SHC cells). MCF-7/IGF-IR clone 17 and MCF-7/IRS-1 clones 9, 3 and 18 were developed by stable transfection of MCF-7 cells with expression vectors encoding either IGF-IR or IRS-1 and have been characterized previously (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). MCF-7/SHC cells are MCF-7-derived cells transfected with the plasmid pcDNA3/SHC; compared with MCF-7 cells, the level of $p55^{SHC}$ and $p47^{SHC}$ overexpression in MCF-7/SHC cells is approximately 5-fold (data not shown). The above MCF-7-derived clones express ERs and respond to E2, similar to MCF-7 cells (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). The levels of IRS-1 in MCF-7/IGF-IR and MCF-7/SHC cells are similar to those in MCF-7 cells (see Fig. 2*b* and data not shown).

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 μg/ml G418. In the experiments requiring E2-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM $FeSO_4$ and 2 mM L-glutamine (PRF-SFM) (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995).

Cell growth assay

Cells were plated at a concentration 2×10^5 in 6-well plates in a growth medium; the following day (day 0), the cells were shifted to PRF-SFM containing different doses of ICI 182,780 (1–300 nM) with or without 50 ng/ml IGF-I and incubated for 4 days. The increase in cell number from day 0 to day 4 in PRF-SFM was designated as 100% growth increase.

IP and WB

The expression and tyrosine phosphorylation of IGF-I signaling proteins were measured by IP and WB, as described previously (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). Protein lysates (500 μg) were immunoprecipitated with the following antibodies (Abs): for the IGF-IR: anti-IGF-IR monoclonal Ab (MAb) alpha-IR3 (Oncogene Science, Cambridge, MA); for IRS-1: anti-C-terminal IRS-1 polyclonal Ab (pAb) (UBI, Lake Placid, NY); for SHC: anti-SHC pAb (Transduction Laboratories, Lexington, KY). Tyrosine phosphorylation was probed by WB with an antiphosphotyrosine MAb PY20 (Transduction Laboratories). The levels of IRS-1, IGF-IR and SHC expression were determined by stripping the phosphotyrosine blots and reprobing them with the following Abs: for IRS-1: anti-IRS-1 pAb (UBI); for IGF-IR: anti-IGF-IR mAb (Santa Cruz Biotechnology, Santa Cruz, CA); for SHC: anti-SHC MAb (Transduction Laboratories). The association of GRB2 or p85 with IRS-1 or SHC was visualized in IRS-1 or SHC blots using an anti-GRB2 MAb (Transduction Laboratories) or an anti-p85 MAb (UBI), respectively. The intensity of bands was measured by laser densitometry scanning.

Northern blotting

The levels of IRS-1 mRNA were detected by Northern blotting in 20 μg of total RNA using a 631 bp probe derived from a mouse IRS-1 cDNA (nt 1351–2002). This fragment (99.8% homology with the human IRS-1 sequence) hybridizes with both human and mouse IRS-1 mRNA (Nishiyama and Wands, 1992).

Statistical analysis

The results in cell growth experiments were analyzed by analysis of variance (ANOVA) or Student's *t*-test, where appropriate.

RESULTS

ICI 182,780 inhibits growth of MCF-7 cells with amplified IGF-IR signaling. Sensitivity to ICI 182,780 is determined by the cellular levels of IRS-1

All cell lines used in this study secrete autocrine IGF-I-like mitogens and are able to proliferate in PRF-SFM (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). The basal (autocrine) growth of the cells was enhanced in the presence of IGF-I (Fig. 1*a,b*). Short (1–2 days) treatments with ICI 182,780 were not sufficient to inhibit cell growth (data not shown), but a 4-day culture in the presence of the antiestrogen produced evident cytostatic effects (Fig. 1*a,b*). In general, the response to ICI 182,780 was dose dependent (Fig. 1*a,b*), however, compared with the other cell lines, the cells highly overexpressing IRS-1 (MCF-7/IRS-1 clones 3 and 18) were more resistant to the drug (Fig. 1*b,c*). Specifically, 1 nM ICI 182,780 inhibited the basal growth by 80, 55 and 50% in MCF-7, MCF-7/IGF-IR and MCF-7/SHC cells, respectively, but the same dose produced only a 20–30% growth inhibition in MCF-7/IRS-1 clones 3 and 18 (Fig. 1*a,b*). Higher concentrations of ICI 182,780 (10 and 100 nM) effectively suppressed the autocrine growth, or even induced cell death in all cell lines, except MCF-7/IRS-1 clone 18, where the maximal reduction (32%) of the basal growth occurred with a 100 nM dose (Fig. 1*b*).

In the presence of IGF-I, the effects of ICI 182,780 were attenuated; 1 nM ICI 182,780 was never cytostatic (data not shown), while the 10 and 100 nM doses inhibited (by 30–50% and 47–78%, respectively) IGF-I-dependent proliferation of cells with low IRS-1 levels (Fig. 1*a,b*). The same doses, however, were less efficient in MCF-7/IRS-1 clones 3 and 18, where growth reduction was 20–25% for 10 nM and 41–47% for 100 nM. Similarly, 300 nM ICI 182,780 produced a prominent cytostatic effect in all cell lines with low IRS-1 expression, but was less active in the clones highly overexpressing IRS-1 (70–93% *vs.* 45–60% growth inhibition) (Fig. 1*a–c*).

The above results suggested that IRS-1 may be an important target for ICI 182,780 action. Consequently, in the next set of experiments we studied the effects of ICI 182,780 on the expression and function of IRS-1.

ICI 182,780 reduces IRS-1 levels and impairs IRS-1 signaling in MCF-7/IRS-1, MCF-7 and MCF-7/IGF-IR cells

In MCF-7/IRS-1 cells grown under basal conditions, IRS-1 was tyrosine phosphorylated for up to 4 days (Fig. 2*a*). IGF-I induced a rapid and marked (5-fold) increase of IRS-1 phosphorylation which persisted for up to 1 day and declined thereafter reaching close to the basal phosphorylation status at day 4. A short ($\leq$1 day) treatment with ICI 182,780 had no consequences on IRS-1 expression or tyrosine phosphorylation. (Fig. 2*a,* panels a and b). However, p85/IRS-1 association was approximately 30% reduced under the basal conditions at day 1 of the treatment (Fig. 2*a,* panel c).

The evident effect of ICI 182,780 action on IRS-1 expression and signaling occurred at day 4, and was especially pronounced in the absence of IGF-I. Specifically, without IGF-I, the drug suppressed IRS-1 protein expression by 60%, which was paralleled by a 60% reduction of IRS-1 tyrosine phosphorylation, and coincided with an almost complete (approximately 95%) inhibition of p85/IRS-1 and GRB2/IRS-1 binding. The addition of IGF attenuated ICI 182,780 action, however, the effects of the treatment remained well detectable: IRS-1 levels were downregulated by 30%, IRS-1 tyrosine phosphorylation by 20% and p85/IRS-1 binding by 30%. Under IGF-I conditions, GRB2/IRS-1 binding was not appreciably affected (Fig. 2*a,* panels a–d).

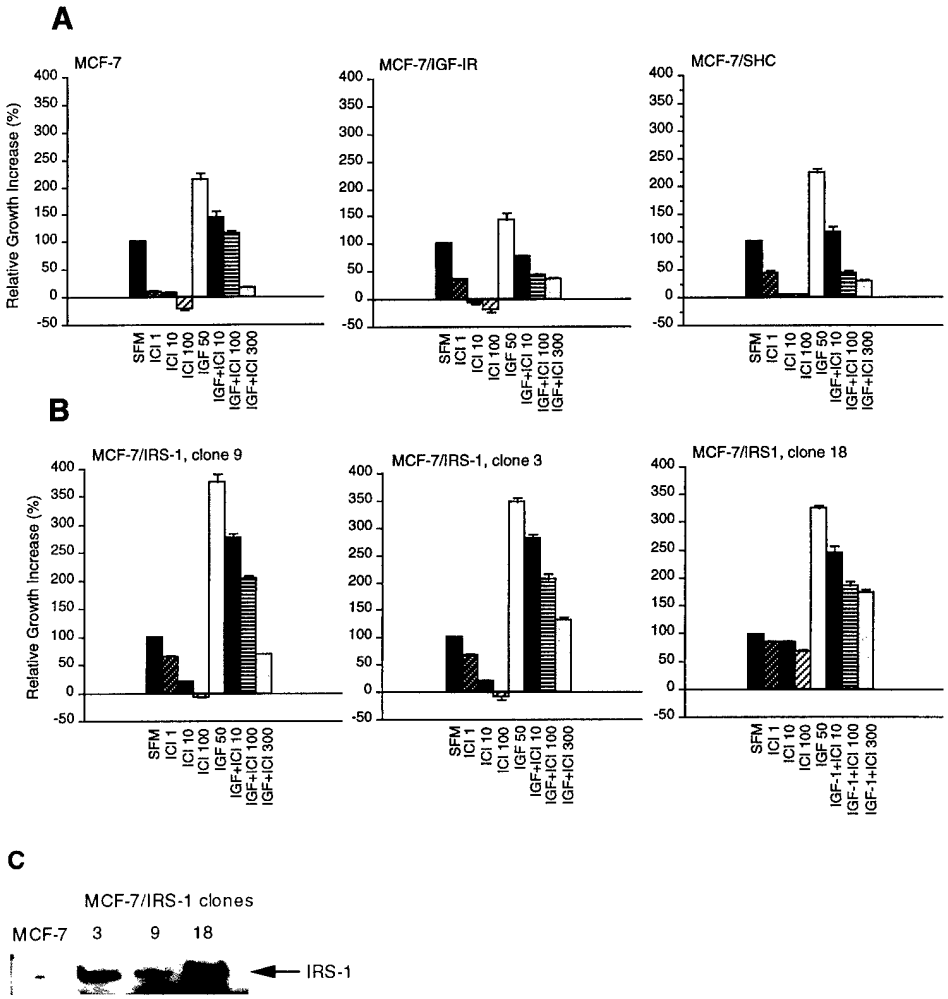

FIGURE 1 – ICI 182,780 inhibits the growth of MCF-7 cells overexpressing different elements of IGF-IR signaling. IRS-1 levels determine ICI 182,780 sensitivity. *(a)* ICI 182,780-induced growth inhibition in the parental MCF-7 cells (8×10^4 IGF-IR/cell), MCF-7/IGF-IR clone 17 (1×10^6 IGF-IR/cell) and MCF-7/SHC (5-fold SHC overexpression over the level in MCF-7 cells). *(b)* Growth reduction in MCF-7/IRS-1 clone 9 (3-fold IRS-1 overexpression over the levels in MCF-7 cells), clone 3 (7-fold overexpression) and clone 18 (9-fold overexpression). The cells were treated with different doses of ICI 182,780 in the presence or absence of 50 ng/ml IGF-I, as described in Material and Methods. The increase in cell number between day 0 and day 4 is taken as 100%. The results are means from at least 4 experiments. Bars indicate standard error. *(c)* Levels of IRS-1 protein in different MCF-7/IRS-1 cell lines. IRS-1 levels were determined by IP and WB as described in Material and Methods. Representative results from 3 experiments are shown.

Importantly, analogous action of ICI 182,780 on IRS-1 expression and tyrosine phosphorylation was seen in other cell lines studied (Fig. 2*b*). In both MCF-7/IGF-IR and MCF-7 cells containing only endogenous IRS-1, ICI 182,780 inhibited the IRS-1 expression under basal conditions by approximately 60%, which was paralleled by the reduced IRS-1 tyrosine phosphorylation (by approximately 90–95%). In the presence of IGF-I, the antiestrogen suppressed the IRS-1 levels by approximately 50% and IRS-1 tyrosine phosphorylation by approximately 40%.

ICI 182,780 attenuates IRS-1 mRNA expression

ICI 182,780 reduced the levels of 5 kb IRS-1 mRNA (Nishiyama and Wands, 1992) in MCF-7 and MCF-7/IGF-IR cells in the absence or presence of IGF-I, by 50 and 70%, respectively (Fig. 3). Moreover, the 5 kb message transcribed from the CMV-IRS-1 plasmid was downregulated (by approximately 70%) in MCF-7/IRS-1 cells treated with both IGF-I and ICI 182,780 (data not shown).

ICI 182,780 inhibits IGF-I-induced but not basal tyrosine phosphorylation of IGF-IR

In MCF-7/IGF-IR cells, IGF-I moderately increased the expression of IGF-IR. This effect was slightly (by 20%) blocked in the presence of ICI 182,780. Under the same conditions, the drug significantly (by 80%) reduced tyrosine phosphorylation of IGF-IR (Fig. 4). ICI 182,780 had no effect on the basal expression of IGF-IR, however, it produced a 30% increase in the basal tyrosine phosphorylation of IGF-IR (Fig. 4). The latter peculiar effect of the antiestrogen occurred in several repeat experiments. Short treatments with ICI 182,780 ($\leq$1 day) were not associated with any significant changes in IGF-IR expression (data not shown).

Long-term ICI 182,780 treatment does not affect SHC signaling

In the presence of IGF-I, SHC tyrosine phosphorylation was moderately induced, with the maximum seen at 1 hr upon stimulation. On the other hand, GRB2/SHC binding peaked at 15 min after IGF-I addition and declined thereafter with the minimal

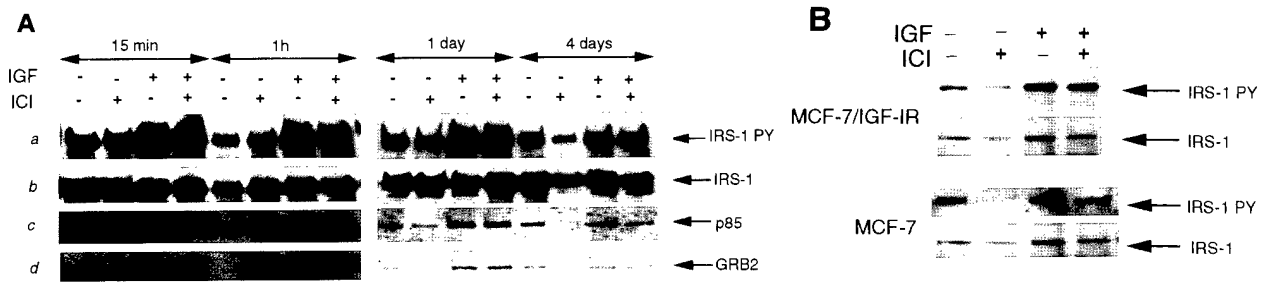

FIGURE 2 – ICI 182,780 inhibits IRS-1-mediated signaling. *(a)* Effects of ICI 182,780 in MCF-7/IRS-1 clone 3. IRS-1 tyrosine phosphorylation (IRS-1 PY) (panel a), protein levels (IRS-1) (panel b), as well as IRS-1-associated p85 of PI-3 kinase (panel c) and GRB2 (panel d) were determined in cells treated for 15 min, 1 hr, 1 day or 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. In the 1 hr treatment, lane IGF (−) ICI (−) is underloaded. Representative results from 5 experiments are shown. *(b)* Effects of ICI 182,780 on IRS-1 in MCF-7/IGF-IR and MCF-7 cells. IRS-1 tyrosine phosphorylation (IRS-1 PY) and protein levels (IRS-1) were examined in cells treated with 100 nM ICI 182,780 for 4 days. Representative blots of 5 experiments are shown.

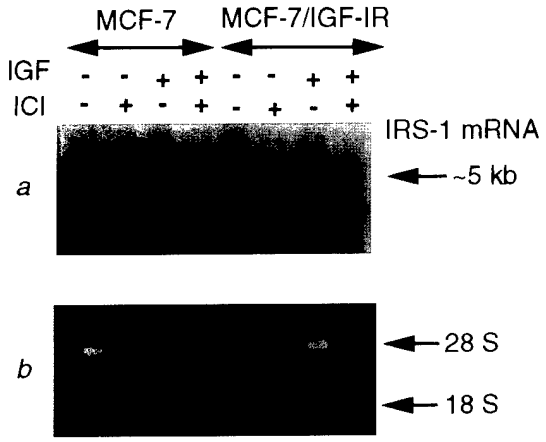

FIGURE 3 – ICI 182,780 attenuates the expression of IRS-1 mRNA levels in MCF-7 and MCF-7/IGF-IR cells. The expression of IRS-1 mRNA was determined in cells treated with 100 nM ICI 182,780 for 4 days in the presence or absence of IGF-I. Panel *a*. IRS-1 mRNA of approximately 5 kb. Panel *b*. Control RNA loading: 28S and 18S RNA in the same blot.

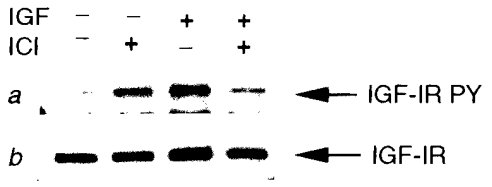

FIGURE 4 – Effects of ICI 182,780 on IGF-IR. IGF-IR tyrosine phosphorylation (IGF-IR PY) (panel *a*) and protein levels (IGF-IR) (panel *b*) in MCF-7/IGF-IR clone 17 treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 3 different experiments are shown.

binding found at day 4 (Fig. 5). ICI 182,780 treatment, in the presence or absence of IGF-I, failed to induce significant changes in the levels or tyrosine phosphorylation of SHC proteins, except a transient stimulation of the basal SHC tyrosine phosphorylation at 15 min (Fig. 5). Importantly, at all time points, SHC/GRB2 association was not influenced by the drug.

Interestingly, at day 4, SHC tyrosine phosphorylation and SHC/GRB2 binding were suppressed in the presence of IGF-I (Fig. 5). This characteristic regulation of SHC by IGF-I, documented by us previously in MCF-7 cells and MCF-7-derived clones, was not affected by ICI 182,780 (Guvakova and Surmacz, 1997).

Similar lack of ICI 182,780 effects on SHC expression and signaling was noted in MCF-7 and MCF-7/IGF-IR cells (data not shown).

DISCUSSION

Pure antiestrogens have been shown to interfere with one of the most important systems regulating the biology of hormone-dependent breast cancer cells, namely, the IGF-I system (de Cupis and Favoni, 1997; Nicholson *et al.*, 1996). The compounds inhibit IGF-induced proliferation, which is associated with, *i.e.*, downregulation of IGF binding sites and reduction of IGF availability. Similar action has been ascribed to non-steroidal antiestrogens such as Tam or 4-OH-Tam (Chander *et al.*, 1993).

The effects of pure antiestrogens on the IGF signal transduction have been unknown. Here, we studied if and how ICI 182,780 modulates the IGF-IR intracellular pathways in breast cancer cells. We focused on the relationship between drug efficiency and signaling capacities of IGF-IR or IRS-1 since these molecules appear to control proliferation and survival in breast cancer cells (Surmacz *et al.*, 1998; Rocha *et al.*, 1997; Turner *et al.*, 1997).

Previously we found that the cytostatic action of Tam involves its interference with IGF signaling pathways. In particular, Tam suppressed tyrosine phosphorylation of IRS-1 and caused hyperphosphorylation of SHC (Guvakova and Surmacz, 1997). The most important conclusion of the present work is that inhibition of IRS-1 expression is an important element of ICI 182,780 mode of action. The first observation was that amplification of IGF signaling did not abrogate sensitivity to ICI 182,780. Next, ICI 182,780 appeared to affect a specific IGF signaling pathway, as the efficiency of the drug was dictated by the cellular levels of IRS-1, but not that of SHC or IGF-IR. For instance, MCF-7/IGF-IR clone 17 was very sensitive to ICI 182,780 despite a 12-fold IGF-IR overexpression, whereas MCF-7/IRS-1 clones 3 and 18 (7- and 9-fold IRS-1 overexpression, respectively) were quite resistant to the drug (Fig. 1). Moreover, ICI 182,780 reduced IRS-1 levels and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I, while its action on IGF-IR was limited to the inhibition of IGF-I-induced tyrosine phosphorylation and its effects on SHC were none.

The reduction of IRS-1 expression by ICI 182,780 occurred in all cell lines studied, however, it was clearly more pronounced in the cells expressing lower (endogenous) levels of the substrate (*i.e.*, MCF-7 and MCF-7/IGF-IR cells) (Fig. 2). This suggests that downregulation of IRS-1 by ICI 182,780 is a saturable process, and overexpression of IRS-1 may provide resistance to the drug. Indeed, although we did not notice a strict correlation between

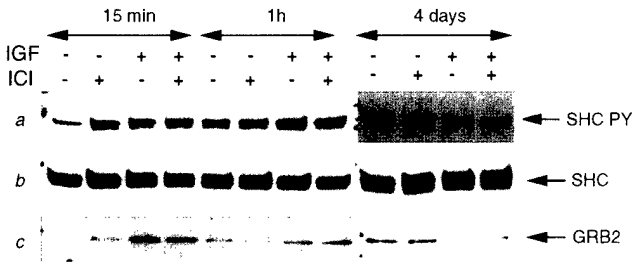

FIGURE 5 – Effects of ICI 182,780 on SHC signaling. SHC tyrosine phosphorylation (SHC PY) (panel *a*), protein levels (SHC) (panel *b*) and SHC-associated GRB2 (panel *c*) were studied in MCF-7/SHC cells treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 5 experiments are shown.

IRS-1 levels or IRS-1 tyrosine phosphorylation and ICI 182,780-dependent growth inhibition, IRS-1-overexpressing cells tended to be more resistant to the cytostatic action of the antiestrogen (Fig. 1). Interestingly, overexpression of IRS-1 clearly had a greater impact on the response to high doses of ICI 182,780 ($\geq$100 nM) than on the effects of low drug concentrations. This suggests that ICI 182,780 action is multiphased, with the initial inhibition being IRS-1 independent (but perhaps, ER-dependent) and the strong growth reduction associated with the blockade of IRS-1 function (Figs. 1, 2).

ICI 182,780 affected IRS-1 expression not only on the level of protein but also on the level of mRNA. In our experiments, the antiestrogen reduced the expression of IRS-1 mRNA in the presence or absence of IGF-I. However, the mechanism by which ICI 182,780 interferes with IRS-1 mRNA expression was not studied here and it remains speculative. Regarding transcriptional regulation, no estrogen-responsive elements have been mapped in the IRS-1 promoter, but it cannot be ruled out that ICI 182,780 acts indirectly through some other regulatory sequences in the 5′ untranslated region of *IRS-1* gene, such as AP1, AP2, Sp1, C/EBP, E box (Araki *et al.*, 1995; Matsuda *et al.*, 1997). A post-transcriptional component may be argued by the fact that the inhibition of IRS-1 mRNA by ICI 182,780 was evident in IGF-I-treated MCF-7/IRS-1 cells, in which the majority of IRS-1 message originated from the expression plasmid devoid of any IRS-1 promoter sequences (CMV-driven IRS-1 cDNA) (Surmacz and Burgaud, 1995) (data not shown). In addition, the finding that ICI 182,780 similarly inhibited IRS-1 mRNA levels under the basal and IGF-I conditions, but IRS-1 protein was significantly more reduced in the absence of IGF-I (Fig. 3 *vs.* Fig. 2*a*) could suggest that the drug acts upon some IGF-I-dependent mechanism controlling mRNA stability, translation, or post-translational events. In fact, in other experimental systems, IGF-I or insulin regulated various messages, including IRS-1 mRNA, on the post-transcriptional level (Araki *et al.*, 1995).

In its action on IRS-1, ICI 182,780 appeared more potent than Tam, which decreased tyrosine phosphorylation of IRS-1 but did not cause any detectable changes in IRS-1 expression. Our results with Tam suggested that this antiestrogen may influence the activity of tyrosine phosphatases (PTPases) (Guvakova and Surmacz, 1997). Indeed, both Tam and ICI 182,780 interfere with IGF-I-dependent growth by upregulating PTPases LAR and FAP-1 (Freiss *et al.*, 1998). In the present work, ICI 182,780 effects on phosphatases acting on IRS-1 were impossible to assess, since the drug also affected IRS-1 expression (Fig. 3). However, some interference of ICI 182,780 with the phosphorylation/dephosphorylation events could be indicated, for instance, by our experiments with IGF-IR, where, under basal conditions, the compound induced IGF-IR phosphorylation without evident modifications of the receptor expression (Fig. 4).

Other important observations stemming from our results concern similarities and differences between the effects of ICI 182,780 and Tam on the IGF-IR and SHC. While Tam did not modulate the expression of IGF-IR protein (Guvakova and Surmacz, 1997), ICI 182,780 moderately decreased IGF-IR levels in the presence of IGF-I. The action of ICI 182,780 and Tam on IGF-IR tyrosine phosphorylation was similar, namely, both compounds inhibited IGF-I-induced but not basal tyrosine phosphorylation of IGF-IR. The effects of ICI 182,780 and Tam on SHC were different. With Tam, we observed elevated tyrosine phosphorylation of SHC proteins and increased SHC/GRB2 binding in growth-arrested cells, while ICI 182,780 did not affect SHC phosphorylation or expression (Guvakova and Surmacz, 1997). Thus, induction of non-mitogenic SHC signaling is a peculiarity of Tam but not a ICI 182,780 mechanism of action.

In summary, cytostatic effects of ICI 182,780, similar to Tam, are associated with the inhibition of IGF-IR signaling. The mitogenic/survival IRS-1 pathway is a target for both antiestrogens. Both drugs reduce the levels of tyrosine phosphorylated IRS-1, but only ICI 182,780 clearly inhibits expression of the substrate. High cellular levels of IRS-1 hinder the response to higher doses of ICI 182,780, thus overexpression of IRS-1 in breast tumors may represent an important mechanism of antiestrogen resistance.

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Role of IRS-1 Signaling in Insulin-Induced Modulation of Estrogen Receptors in Breast Cancer Cells

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Cross-talk between steroid hormones and polypeptide growth factors regulates the growth of hormone-responsive breast cancer cells. For example, in the MCF-7 human breast cancer cell line, insulin up-regulates estrogen receptor (ER) content and binding capacity. Since the insulin receptor (IR) substrate 1 (IRS-1) is one of the core signaling elements transmitting mitogenic and metabolic effects of insulin, we investigated whether IRS-1 is also required for the insulin-induced function of the ER. The effects of insulin on the ER were compared in MCF-7 cells and MCF-7-derived cell lines with decreased levels (by ~80%) of IRS-1 due to the expression of IRS-1 antisense RNA. The severe IRS-1 deficiency in MCF-7 cells was associated with (1) reduced mitogenic response to 20 ng/ml insulin and 10% calf serum (CS), but not to 1 nM estradiol (E2); (2) loss of insulin-E2 synergism; (3) up-regulation of ER protein expression and binding capacity; and (4) loss of insulin-induced regulation of ER tyrosine phosphorylation. In conclusion, the data confirm the existence of the IR–ER cross-talk and suggest that IRS-1-dependent signaling may contribute to the negative regulation of the ER expression and function in MCF-7 cells.

The growth of hormone-responsive breast cancer cells *in vitro* and *in vivo* is controlled by steroids and polypeptide growth factors (1, 2). Accumulating evidence indicates that this growth control involves complex interactions, or cross-talk, between the two mitogenic systems. For instance, E2 stimulates the expression of several growth factors [such as insulin-like growth factors (IGFs), transforming growth factor alpha and beta, and amphiregulin], alters the levels or activity of different growth factor receptors [such as IGF-I and IGF-II receptors, and the epidermal growth factor (EGF) receptor], as well as modulates the expression of different IGF binding proteins (1–4). E2 is also able to modulate intracellular growth factor signaling pathways. For example, one of the acute effects of E2 binding to the ER in MCF-7 cells is stimulation of c-src tyrosine kinase and activation of c-src-dependent signaling substrates, including SHC (src-homology/collagen protein), which in consequence induces classic growth factor-responsive Ras/MAP (mitogen-activated kinase) cascade of kinases (5, 6). In addition, antiestrogens can block mitogenic action of growth factors on breast cancer cells through an ER-dependent or -independent mechanism (1–4, 7).

The other significant element of the cross-talk is modulation of ER expression and function by polypeptide growth factors (8–10). For example, different peptide mitogens can stimulate ER transcriptional activity even in the absence of E2 (11–17), probably through the phosphorylation of the ER on critical residues of Ser 118 and Tyr 537 (10, 16, 18). We have previously demonstrated that in MCF-7 cells, insulin up-regulates ER content and ER binding capacity, which is blocked in the presence of tyrosine kinase inhibitor, genistein (9). The intracellular signaling mechanism by which insulin regulates ER in breast cancer cells is not known. One of the major signaling pathways of the IR involves a substrate, IRS-1 (19). IRS-1 is a docking protein which becomes phosphorylated on multiple tyrosine residues immediately upon insulin binding to the IR. Tyrosine phosphorylated IRS-1 associates with different SH2-domain containing proteins activating a spectrum of downstream signaling pathways, such as Ras/MAP or PI-3 kinase pathways (19). The critical role of IRS-1 signaling in metabolic and mitogenic action of insulin has been well established in many cellular systems (19–21). In MCF-7 cells, IRS-1 is re-

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quired for monolayer and anchorage-independent growth, and is critical for transmitting signals controlling cell survival (21, 22). In this study, we examined the role of IRS-1 in IR-ER cross-talk.

MATERIALS AND METHODS

Cell lines. MCF-7/anti-IRS-1 clones 2 and 9 have been generated by stable transfection of MCF-7 cells with an expression plasmid encoding antisense IRS-1 RNA. The IRS-1 protein levels in MCF-7/anti-IRS-1 clones 2 and 9 have been down-regulated by approximately 80% and 85%, respectively. The other characteristics of these cells have been previously described in Ref. 22. In all experiments, the parental MCF-7 cells were used as a control. The clone overexpressing IRS-1 (MCF-7/IRS-1, clone 3) was obtained by stable transfection of MCF-7 cells with the CMV-IRS-1 plasmid that contains a mouse IRS-1 cDNA cloned into the Hind III site of the pRC/CMV mammalian expression vector (Invitrogen) (21). The resulting plasmid also confers neomicin resistance (inherent in pRC/CMV).

Routine cell culture. MCF-7 cells were grown in DMEM:F12 supplemented with 5% calf serum (CS); MCF-7/anti-IRS-1 cells were cultured in DMEM:F12 plus 5% CS plus 200 mg/ml G418 (22).

Experimental culture conditions. The cells cultured in growth medium were trypsinized and plated in phenol red-free (PRF) DMEM:F12 supplemented with 5% dextran-coated charcoal stripped CS (DCC-CS medium). After 24 h, this medium was changed to PRF-DMEM:F12 for another 24 h. Next, the cells synchronized in PRF-DMEM:F12 (day 0) were treated for 1, 2, or 96 h with PRF-DMEM:F12 containing 10% of dithiotreitol treated DCC-CS (DCC-SH-CS medium) supplemented with 20 ng/ml of insulin or 1 nM E2. DCC-CS and DCC-SH-CS were prepared as previously described (23, 24).

DNA content. Cells were plated in 24-well plates at a density of 1.5×10^4 cells/cm^2 in DCC-CS medium and then shifted to PRF-DMEM:F12 and DCC-SH-CS, as described above. At different times of treatment, cellular DNA content per well was assessed by fluorescent staining with Hoechst 33258, as described previously (9).

Estrogen receptor binding assay. ER binding sites in cytosol and nuclear fractions were determined by Scatchard analysis with ^{3}H-E2, as previously described (9). In brief, the cells were seeded at a density of 1.13×10^4/cm^2 in 100-mm culture plates in growth medium, then shifted to DCC-CS, PRF-DMEM:F12 and next to DCC-SH-CS, as described above. The synchronized cells were treated with mitogens for different times. Cytosol fractions were obtained by harvesting and sonicating the cells in a cytosol buffer (9). The sonicate was sedimented at 15,000 g for 30 min at 4°C. The supernatant contained the cytosol fraction, whereas the pellet contained cell nuclei.

Cytosol ER content. 50 ml of the cytosol fraction were incubated for 18 h at 4°C with 0.125–4 nM ^{3}H-E2. Non-specific binding was determined by incubating the cells with ^{3}H-E2 in the presence of 500-fold molar excess of diethylstilbestrol (DES). Bound and free E2 were separated by absorbing free hormones on DCC (100 μl) at 4°C for 15 min. The radioactivity of the bound hormone was determined in a scintillator counter.

Nuclear ER content. The nuclear pellet (obtained as described above) was resuspended in 0.6 M KCl for 1 h at 4°C, and then centrifuged at 15,000 g for 30 min at 4°C. Fifty microliters of the supernatant (nuclear fraction) was incubated with 2–64 nM of ^{3}H-E2 for 18 h at 4°C. The non-specific binding was determined with a 250-fold molar excess of DES.

Western blotting and immunoprecipitation. The protein levels and tyrosine phosphorylation of the ER were measured by immunoprecipitation (IP) followed by Western Blotting (WB), as previously described (7, 9). Following treatment, the cells were lysed in ice-cold lysis buffer (9). ER protein levels were determined by IP 500 μg of protein lysate with an anti-ER 304 antibody (Ab) (Neo Marker, Freemont USA), followed by WB using an anti-ER 311 Ab (Neo Marker, Freemont USA). CHO (ER-negative) cells were used as a negative control. Tyrosine phosphorylation of the ER was detected by immunoprecipitating 500 μg of protein lysate with an anti-ER 304 Ab (Neo Marker, Freemont USA), followed by immunoblotting with an anti-phosphotyrosine monoclonal Ab (mAb) PY20 (Santa Cruz Biotechnology, CA).

Statistical analysis. Data were analyzed using analysis of variance (ANOVA) or paired t-test, where applicable.

RESULTS

Down-regulation of IRS-1 inhibits growth-promoting effects of serum and insulin but not that of E2. The requirement for IRS-1 in IR-induced effects on the ER was studied using two MCF-7/anti-IRS-1 clones (2 and 9), in which the levels of IRS-1 were down-regulated by 80 and 85%, respectively. First, we investigated how decreased levels of IRS-1 affect growth response (measured as an increase of DNA content) to different mitogens. Figures 1A and 1B illustrates growth-promoting effects of 10% CS and 20 ng/ml insulin in MCF-7 and MCF-7/anti-IRS-1 cells synchronized in PRF-DMEM:F12. The mitogenic response to 10% CS in MCF-7/anti-IRS-1 clone 2 and 9, was suppressed by 55 (±SE 0.0) % and 57(±SE 0.0)%, respectively, compared that in the parental cells (Fig. 1A). Importantly, 20 ng/ml insulin did not produce any significant increase in DNA content in both MCF-7/anti-IRS-1 clones, whereas it stimulated DNA synthesis in the parental cells [40 (±SE 9.0)%; $p < 0.05$; Fig. 1B].

In contrast, the growth-promoting effect of 1 nM E2 in all cell lines was similar (Fig. 1B; variation among the cell lines: p = NS). In particular, in MCF-7 cells, E2 stimulation resulted in a 55 (±SE 11.1)% augmentation in DNA content over the basal level, and in MCF-7/anti-IRS-1, clones 2 and 9, the increase was 56 (±SE 15.3)% and 56 (± SE 11.2)%, respectively (Fig. 1B).

The stimulation of DNA synthesis by a combination E2 plus insulin was synergistic in all cell lines, but quantitatively smaller in MCF-7/anti-IRS-1 cells ($p < 0.05$, Fig. 1B) Specifically, whereas under the treatment, in MCF-7 cells DNA content increased 187 (±SE 40.0)%, in MCF-7/anti-IRS-1 clone 2 and 9, the stimulation was 142 (±SE 39.0)% and 88 (±SE 18.2)%, respectively.

Down-regulation of IRS-1 in MCF-7 cells is accompanied by the increase in both ER protein levels and ER binding capacity. We tested ER protein expression and binding capacity in MCF-7/anti-IRS-1 cells and, for comparison, in MCF-7/IRS-1 clone 3. All these cell lines express a neomycin resistance gene thus allowing us to test the eventual interfering effect of this gene on the ER. The basal content of the ER was clearly enhanced in both MCF-7/anti-IRS-1 clones with respect to MCF-7 cells. Interestingly, a 9-fold overexpression of IRS-1 in MCF-7/IRS-1, clone 3 did not significantly modulate ER expression (Figs. 2A and 2B). Scatchard

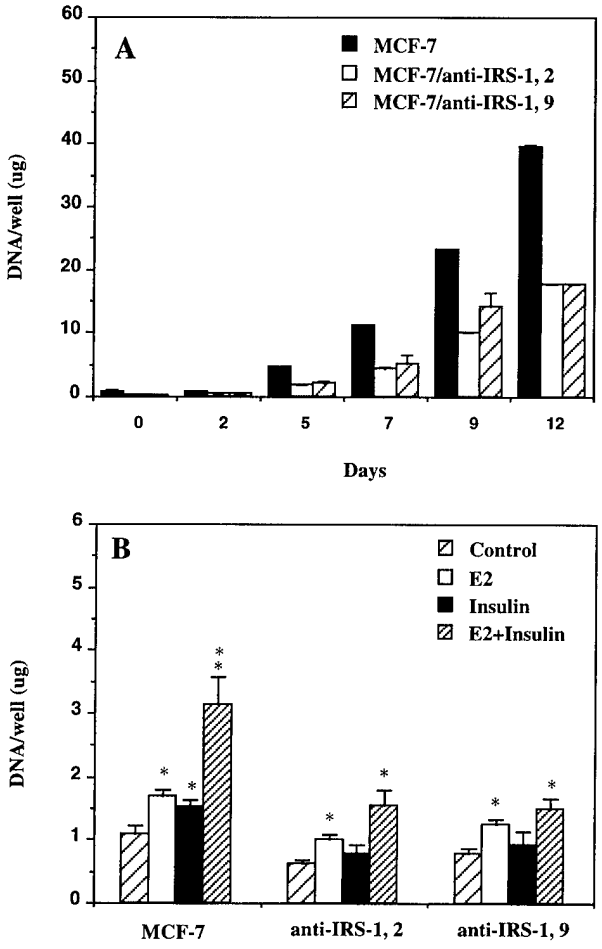

FIG. 1. Mitogenic effects of (A) 10% CS on MCF-7 and (B) Estradiol (E2), insulin or a combination of E2 and insulin on MCF-7 and MCF-7/anti-IRS-1 cells. The cells were synchronized in DCC-SH-CS medium (Day 0) and cultured in the presence of either 10% CS, 1 nM E2, 20 ng/ml insulin or 1 nM E2 plus 20 ng/ml insulin for 2–12 days. Cell DNA content was determined as described in Materials and Methods. *$p < 0.05$; **$p < 0.01$ versus control (DNA content at day 0).

analysis of E2 binding sites upon insulin stimulation confirmed significant upregulation of ER content in MCF-7/anti-IRS-1 clones compared with MCF-7 and MCF-7/IRS-1 cells (Fig. 3).

IRS-1 levels impact the regulation of basal and insulin-induced tyrosine phosphorylation of the ER. The regulation of the ER protein expression and tyrosine phosphorylation by insulin and E2 was determined in MCF-7 cells and in MCF-7/anti-IRS-1, clone 2 (Fig. 4). In the parental cells, the expression of the ER was elevated with both mitogens at 12 h and 96 h of treatment. In contrast, MCF-7/anti-IRS-1 cells expressed high basal levels of the ER that appeared refractory to insulin regulation, and were reduced by E2.

The basal ER tyrosine phosphorylation at 12 h was significantly elevated in MCF-7/anti-IRS-1 cells, compared with that seen in MCF-7 cells. Moreover, whereas in MCF-7 cells, insulin and E2 up-regulated ER phosphorylation, in MCF-7/anti-IRS-1 cells, these treatments had no effect on the ER phosphorylation status (Fig. 4B). At 96 h, basal ER tyrosine phosphorylation was similar in both cells lines, but it was clearly down-regulated by insulin in MCF-7 cells and not affected in MCF-7/anti-IRS-1 cells. E2 at 96 h induced the phosphorylation of the ER in both cell lines.

DISCUSSION

Cross-talk between signaling pathways of the IR or the IGF-IR and the ER is a powerful mechanism controlling the growth of many hormone-responsive breast cancer cells. It is thought that deregulation of this cross-talk may lead to the development of hormone-independence and antiestrogen-resistance (1, 3, 4). The molecular basis of growth factor-steroid communication are not entirely clear. Although considerable knowledge exists about the effects of estrogens on IGF or insulin systems, the role of these growth factors on ER expression and function is less known. In this context, of particular interest are recent data demonstrating that IGF or insulin are able to up-regulate E2 binding sites and stimulate the transcription of E2-responsive DNA, even in the absence of E2 (8, 9, 11, 17). In the latter case, growth factors appear to induce phosphorylation of the unligated ER on Ser 118 (via MAP kinase pathway), and possibly on Tyr 537, in consequence enhancing ER transcriptional activity (10, 18, 25). These observations, together with our present results, provide the evidence that the IR modulates ER function on at least three different molecular levels: a) ER protein expression; b) ER binding capacity; and c) ER phosphorylation. The post-receptor events involved in the control of the ER by insulin are

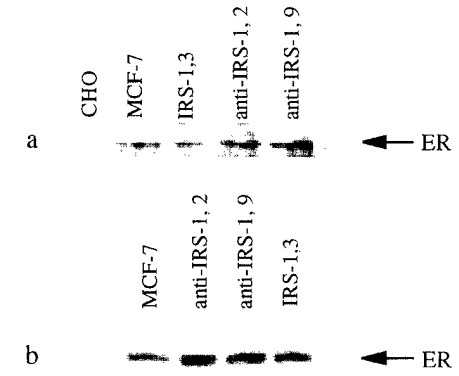

FIG. 2. ER protein content in growing MCF-7 cells, MCF-7/anti-IRS-1, clones 2 and 9, and in MCF-7/IRS-1, clone 3. ER protein content was determined in whole cell lysates (a) by WB or by IP followed by WB with specific anti-ER Ab (b) as described in Materials and Methods.

still poorly characterized. Here, we examined the role of IRS-1, a principal substrate of the IR that is critical for its metabolic and mitogenic action, in the modulation of ER expression and tyrosine phosphorylation.

First, IRS-1 was not critical for the stimulation of ER binding capacity by insulin, since in MCF-7 cells with significantly (~80%) decreased levels of IRS-1, insulin normally up-regulated E2 binding sites (Fig. 3), whereas its growth-promoting action was inhibited under the same conditions (Fig. 1B). The possibility that other IR-dependent signaling pathways, such as SHC or PI-3 kinase pathways, are responsible for stimulating E2 binding sites in MCF-7 cells is currently under investigation.

Second, IRS-1 signaling may contribute to a physiological down-regulation of ER protein levels in MCF-7 cells, as the reduction of IRS-1 levels was clearly paralleled by an increase of ER expression and binding capacity (Fig. 1B, 2, and 4). However, overexpression of IRS-1 did not reduce ER levels, which suggest that the regulation of the ER depends on some other signaling pathways. Why lower levels of IRS-1 trigger ER overexpression is not known. Perhaps, when IR-dependent mitogenicity is compromised, as occurred in MCF-7/anti-IRS-1 cells, a compensatory mechanism stimulates an overexpression of the ER. Interestingly, however, this ER overexpression in MCF-7/anti-IRS-1 clones was not reflected by an increased mitogenic response to E2 (Fig. 1B), indicating that stimulation of cell growth by E2 is a saturable process, possibly controlled by a negative effect of estradiol on its own receptor (Ref. 26 and Fig. 4A).

Third, our studies suggest that IRS-1 is important for the regulation of ER tyrosine phosphorylation, at least in cells exposed to insulin for 96h. Specifically, such a long-term insulin treatment evidently reduced the ER phosphorylation in the parental cells, but it produced no effect in MCF-7/anti-IRS-1 cells.

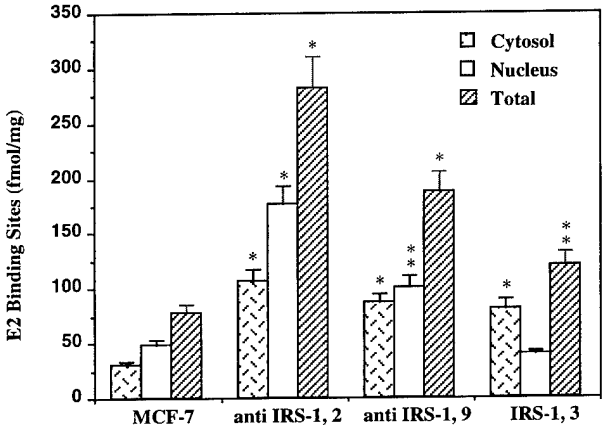

FIG. 3. Binding capacity of cytosolic, nuclear and total ER under basal conditions and upon insulin (20 ng/ml) stimulation in MCF-7 cells, MCF-7/anti-IRS-1 clones 2 and 9, and in MCF-7/IRS-1, clone 3. *$p < 0.05$; **$p < 0.01$ versus control.

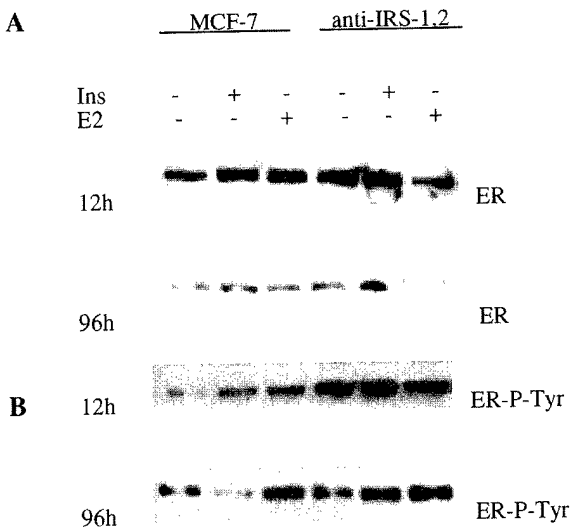

FIG. 4. (A) E2 protein content (ER) in MCF-7 and in MCF/anti-IRS-1, clone 2. The levels of ER in cells untreated or treated with either insulin (20 ng/ml) or estradiol (1 nM) were determined by IP and WB at 12 and 96 h. (B) Tyrosine phosphorylation of the ER (ER-P-Tyr) in MCF-7 and MCF-7/anti-IRS-1 clone 2 in cells untreated or treated with either insulin (20 ng/ml) or E2 (1 nM) for 12 or 96 h. ER content and ER tyrosine phosphorylation were determined by stripping the blots from Fig. 4A. and reprobing with an anti-phosphotyrosine mAb as described under Materials and Methods.

The biological relevance of ER tyrosine phosphorylation is still under debate. It is possible that overall tyrosine phosphorylation of the ER is not directly related to E2 transcriptional and growth effects, as already suggested by other investigators; for example ER phosphorylation has been shown to be induced by both estrogen and antiestrogens (11). The concept that in our system, impaired IRS-1 signaling affected phosphorylation of the ER on Ser 118, in consequence reducing ER transcriptional activity is currently under investigation.

In summary, IRS-1 pathway appears to be required for IR-dependent proliferation in MCF-7 cells, but not for E2-stimulated growth. In addition, the data suggest that IRS-1 may contribute to the process of physiologic downregulation of ER expression and function.

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Type I insulin-like growth factor receptor function in breast cancer

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Key words: breast cancer, IGF-I receptor, IRS-1, SHC, adhesion, apoptosis

Summary

Experimental evidence suggests an important role of the type I IGF receptor (IGF-IR) in breast cancer development. Breast tumors and breast cancer cell lines express the IGF-IR. IGF-IR levels are higher in cancer cells than in normal breast tissue or in benign mammary tumors. The ligands of the IGF-IR are potent mitogens promoting monolayer and anchorage-independent growth of breast cancer cells. Interference with IGF-IR activation, expression, or signaling inhibits growth and induces apoptosis in breast cancer cells. In addition, recent studies established the involvement of the IGF-IR in the regulation of breast cancer cell motility and adhesion. We have demonstrated that in MCF-7 cells, overexpression of the IGF-IR promotes E-cadherin-dependent cell aggregation, which is associated with enhanced cell proliferation and prolonged survival in three-dimensional culture.

The expression or function of the IGF-IR in breast cancer cells is modulated by different humoral factors, such as estrogen, progesterone, IGF-II, and interleukin-1. The IGF-IR and the estrogen receptor (ER) are usually co-expressed and the two signaling systems are engaged in a complex functional cross-talk controlling cell proliferation.

Despite the convincing experimental evidence, the role of the IGF-IR in breast cancer etiology, especially in metastatic progression, is still not clear. The view emerging from cellular and animal studies is that abnormally high levels of IGF-IRs may contribute to the increase of tumor mass and/or aid tumor recurrence, by promoting proliferation, cell survival, and cell-cell interactions. However, in breast cancer, except for the well established correlation with ER status, the associations of the IGF-IR with other prognostic parameters are still insufficiently documented.

Introduction

The IGF-IR is ubiquitously expressed in human tissues and has been implicated in diverse biological processes, such as mitogenesis, cellular transformation, survival, and differentiation [1]. The important function of the IGF-IR in the biology of normal mammary gland has been well established [2]. There is also experimental evidence suggesting the involvement of the IGF-IR in the development of breast cancer [2,3]. In particular, the IGF-IR has been found significant-

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Table 1. Correlation of the IGF-IR with prognostic parameters and selected host or tumor variables.

Parameter	No. cases / % IGF-IR positive	Method of IGF-IR assessment	Correlation with IGF-IR	References
age	224 / 80%	RIA	NS	[6]
	126 / 39%	binding	NS	[9]
	599 / 87%	binding	positive	[4, 10]
	214 / 93%	binding	positive	[11]
	171 / 83%	binding	NS	[12]
ER	224 / 80%	RIA	positive	[6]
	126 / 39%	binding	positive	[9]
	599 / 87%	binding	highly positive	[4]
	214 / 93%	binding	positive	[11]
PgR	224 / 80%	RIA	NS	[6]
	126 / 39%	binding	NS	[9]
	214 / 93%	binding	weakly positive	[4]
	171 / 80%	binding	positive	[12]
IR	224 / 80%	RIA	positive	[6]
DFS	126 / 39%	binding	negative	[9]
	599 / 87%	binding	positive	[4]
	214 / 93%	binding	NS	[11]
OS	599 / 87%	binding	positive	[4]
	214 / 93%	binding	NS	[11]

NS=not significant; only studies with series of 100 or more tumor samples are included in the table.

ly overexpressed in cancer cells with respect to its status in normal breast epithelium or in benign tumors [4-6]. This last observation raised several important questions. First, can IGF-IR be recognized as a prognostic factor in breast cancer? Second, what are the molecular bases of IGF-IR contributions to the development and progression of breast cancer? And third, is the IGF-IR, or its signaling pathway, an appropriate target for endocrine therapy?

IGF-IR in breast tumors. Correlation with other variables. Prognostic value of the IGF-IR

The studies on IGF-IR expression in breast tumors and its correlation with other host or tumor parameters are very limited. Moreover, the interpretation of the available data is complicated by the fact that different techniques were used to assess the IGF-IR levels (Table 1). The most frequently performed IGF-I binding assay is known to be inherently inaccurate due to the interaction of IGF with membrane IGFBPs, which often results in overestimation of the number of IGF-IR [7]. To alleviate this problem, several laboratories examined the expression of IGF-IR mRNA by Northern blotting or RNase protection [2-4]. These methods, however, cannot be used to quantitate the levels of IGF-IR protein. The most recent analyses of breast cancer specimens relied on specific methods, such as RIA or immunocytochemistry, both employing anti-IGF-IR antibodies [6,8].

Despite the differences in experimental approach, the expression of the IGF-IR in breast tumors has been unequivocally established by all large series studies (>100 samples) (Table 1) [4,6,9-12]. Most important, IGF-IR levels have been found elevated in breast cancer compared with non-malignant tumors or normal epithelium [4,6,13]. The mechanism of the common IGF-IR overexpression in breast cancer is not clear, but

it does not appear to be related to IGF-IR gene amplification [14].

The attempts to correlate IGF-IR expression with other host or tumor variables determined a positive link between the IGF-IR and ER status (Table 1) [6,9,4,11]. In addition, one study documented frequent co-expression of the IGF-IR and the insulin receptor (IR) [6]. Several large series analyses found no significant correlations between IGF-IR expression and menopausal status [6], body weight [6], tumor size [4,6,12], tumor grade [6,9,12], histological type [4,6], node status [4, 6,9,11], or epidermal growth factor receptor (EGFR) status [12]. The data on the correlation with age are conflicting [4,6,9-12]. Similarly, still unclear is the association of IGF-IR with progesterone receptor (PgR) status [6,9,11,12] (Table 1).

The IGF-IR, taken as an independent factor, has been reported as either a positive, a negative, or an insignificant marker of disease-free survival (DFS) [4,9,11]. Likewise, the data concerning the link between overall survival (OS) and the IGF-IR remain inconclusive [4,11] (Table 1). A recent study assessed IGF-IR status in two patient subgroups, representing either a low risk (ER^+, PgR^+, low mitotic index, diploid) or a high risk (ER^-, PgR^-, high mitotic index, aneuploid) population. Notably, this analysis established a highly significant correlation between IGF-IR expression and better prognosis [5,6].

In addition, in several small series studies, the following interesting associations have been reported: node-positive/IGF-IR^+ tumors appeared to bear worse prognosis than node-negative/ IGF-IR^+ tumors [9]; DFS was found to be shorter in ER^-/IGF-IR^+ than in ER^+/IGF-IR^+ tumors [9]; in 43 samples of locally recurring tumors, high IGF-IR levels (by immunocytochemistry) correlated with resistance to radiotherapy (P. Glazer, Yale University, personal communication); in 11 cases undergoing primary chemotherapy, the IGF-IR level (by immunocytochemistry) decreased, while that of Bcl-2 increased [8]. Moreover, high levels of IGF-I binding sites correlated with an increased expression of p53, based on the analysis of 17 cases [15]. In 13 samples of metastatic breast cancer, the levels of the IGF-IR were found comparable to those in the primary tumor [12].

Despite the evident complexity and insufficiency of clinical data, over the recent years our understanding of IGF-IR's role in breast cancer has significantly expanded through studies using animal and cellular models. Additional information has emerged from the work determining the mechanism of IGF-IR signal transduction and the biological significance of different signaling pathways of this receptor in breast cancer cell biology.

IGF-IR signaling

The IGF-IR belongs to the tyrosine kinase receptor superfamily (type I receptors) and shares 70% homology with the IR [16]. Both receptors are often co-expressed in breast cancer cells, which promotes the formation of IGF-IR/IR hybrids exhibiting high affinity to IGF-I and, most probably, signaling through the IGF-IR pathway [6,17].

Activation of the IGF-IR results in its oligomerization, autophosphorylation, and activation of the intrinsic tyrosine kinase [16]. The resulting folding of receptors aids the recruitment of several cellular substrates, which then couple the IGF-IR to downstream signaling pathways by serving as binding sites for other effector proteins [1]. Over the past several years, the following substrates of the IGF-IR have been identified: insulin receptor substrate 1 (IRS-1) [18], IRS-2 [19], src-homology 2/collagen alpha proteins (SHC) [20,21], phosphatidyl inositol-3 kinase (PI-3K) [22], growth factor receptor binding protein 10 (GRB-10) [23] and its splice variant GRB10/IR-SV1 [24].

The best characterized IGF-IR signaling molecules are IRS-1 and SHC. Both of these substrates become rapidly tyrosine phosphorylated by the activated IGF-IR, which endows them with the ability to associate with different SH2-containing proteins and to stimulate multiple pathways. For instance, IRS-1 activates PI-3K (through the

Table 2. Inhibition of breast cancer cell growth by interference with the IGF-IR expression or IGF-IR intracellular signaling.

Strategy	Effect	References
Antisense RNA to IGF-IR	Reduced IGF-IR levels; inhibition of monolayer growth	[40]
IGF-IR dominant negative mutant	Reduced anchorage-independent growth	Surmacz et al., this paper
Antisense RNA to IRS-1	Reduced IRS-1 levels; inhibition of anchorage-dependent and -independent growth; apoptosis.	[42]
Antisense oligos to IRS-1	Reduced IRS-1 tyrosine phosphorylation; inhibition of anchorage-dependent and -independent growth.	[34]
Antisense RNA to SHC	Reduced SHC protein levels; inhibition of anchorage-dependent and -independent growth; reduced cell motility and aggregation on Matrigel	[42]
Tamoxifen	Inhibition of IRS-1 tyrosine phosphorylation; restriction of growth.	[50,64]
Il-1	Inhibition of IGF-IR tyrosine phosphorylation; suppressed monolayer growth	[63]

Inhibition of IGF-IR activation by interference with ligand availability has been omitted from this table.

association with the p85 regulatory subunit of PI-3K), Ras/MAP cascade (through GRB2/SOS), SYP phosphatase, as well as other more obscure pathways involving adapters Nick and Crk [18, 25]. Tyrosine phosphorylated SHC, like IRS-1, recruits GRB/SOS complexes and activates the Ras/MAP pathway [20,21]. Most, if not all, elements of IGF-IR signaling are shared with other systems; for instance, IRS-1 is activated by the IR as well as different non-tyrosine kinase receptors (via Janus-type kinases [18,26]). SHC is a common substrate of most tyrosine kinase receptors, cytoplasmic tyrosine kinases, and certain phosphatases [20,21,27,28]. In addition, both IRS-1 and SHC are known to associate with molecules involved in cell-cell and cell-substrate adhesion [29-32]. Hence, cellular response to IGF-I most definitely depends not only on the number of available IGF-IRs, but also on cellular context, and is subject to modification by different extracellular stimuli.

IGF-IR and breast cancer cell growth

The ligands of the IGF-IR, IGF-I and IGF-II (IGFs) as well as insulin at superphysiological concentration, are potent mitogens for breast cancer cells in monolayer culture [2,3]. In breast cancer cell lines, the IGF-IR is often co-expressed with autocrine IGF-like mitogens [33], promoting proliferation under conditions of reduced growth factors [29,33,34]. The growth of breast tumors *in vivo* appears to be regulated in a more complex way, involving locally secreted (paracrine and autocrine) and possibly circulating IGFs [2,3,35].

A critical role of the IGF-IR in breast cancer growth has been confirmed by different strategies interfering with the receptor function. Several studies demonstrated that a blockade of the IGF-IR with an anti-IGF-IR antibody inhibited the proliferation of different breast cancer cell lines *in vitro* and the growth of some xenografts in nude mice [36,37]. The effective inhibition of IGF-IR-mediated growth was also accomplished through the reduction of ligand availability. For instance, exposure of breast cancer cells to excess IGF-BP1 or suramin limited IGF-IR stimulation [38,39]. Interference with the synthesis of IGF-IR provided additional evidence. In MCF-7 cells, the expression of an anti-IGF-IR RNA reduced IGF-IR mRNA by ~30% and IGF-IR protein by ~40%. These effects were accompanied by growth inhibition in monolayer culture [40] (Table 2).

The requirement for IGF-IR in anchorage-independent growth of breast cancer cells has been well documented with the use of anti-IGF-IR antibodies [37,41]. In addition, our recent studies demonstrated that in MCF-7 cells, normal levels of IRS-1 and SHC proteins are required to support growth in soft agar [42]. To further study the function of IGF-IR signaling in transformation, we generated MCF-7/IGF-IR/TC cells expressing a truncated mutant of IGF-IR lacking the C-terminal domain (IGF-IR/TC). This mutant receptor transmits a normal mitogenic signal but has impaired transforming abilities (growth in soft agar) when expressed in murine fibroblasts [43]. In several MCF-7-derived clones, each expressing approximately $1x10^5$ IGF-IR/TC, we found that anchorage-independent growth was inhibited by 42-70%, whereas monolayer growth was not affected (Table 2). These preliminary results demonstrate that in breast cancer cells, as in fibroblasts, transformation depends on a signaling pathway originating at the C-terminal region of the IGF-IR. The identity of this pathway is under investigation.

IGF-IR overexpression and mitogenicity in MCF-7 cells

Further understanding of IGF-IR function in the pathobiology of breast cancer cells has been provided by the recently developed cellular models. Specifically, Daws et al. and our laboratory generated MCF-7-derived clones overexpressing different levels of the IGF-IR (MCF-7/IGF-IR cells) [29,44]. The extent of IGF-IR overexpression was 3.5–4.5-fold (Daws et al.) and 8–50-fold (our laboratory) over the level in MCF-7 cells. In both cases, the overexpression of the IGF-IR in MCF-7 cells produced surprisingly moderate mitogenic effects (measured by ^{3}H-thymidine incorporation). In particular, the sensitivity to IGF-I in MCF-7/IGF-IR cells was unchanged, even in cells with 50-fold receptor overexpression. Moreover, the responsiveness of MCF-7/IGF-IR cells to IGF-I was comparable to that of the parental cells, or only moderately increased (maximum 2-fold) in cells with the highest IGF-IR levels [29]. In the presence of 10 nM estradiol (E2), the MCF-7/IGF-IR clones exhibited an increased responsiveness to low concentrations of IGF-I (0.1-1.0 ng/ml), but with higher concentrations of IGF-I, the synergistic effect was abolished, and the maximal stimulation of DNA synthesis was achieved with IGF-I alone. In the study of Daws et al., E2 combined with high amounts of IGF-I (50ng/ml) inhibited monolayer growth of cells overexpressing the IGF-IR [44], while we noted that the growth rate of MCF-7/IGF-IR clones cultured in PRF-SFM containing either 50 ng/ml IGF-I or 5% calf serum was comparable to that of MCF-7 cells [29]. The anchorage-independent growth of MCF-7/IGF-IR was slightly enhanced in E2 [44], but not in PRF-SFM or medium supplemented with 5%CS [29].

The above data suggest that, in breast cancer cells, overexpressed IGF-IRs sensitize cells to very low amounts of ligands, especially in the presence of estrogens, and could promote cell growth under conditions of limited ligand availability. However, IGF-IR-dependent mitogenicity also appears to be a saturated process, strictly controlled by the ER system.

IGF-IR and E-cadherin-dependent adhesion

In contrast with the minimal consequences of IGF-IR overexpression on the monolayer growth of MCF-7 cells, the amplification of the IGF-IR produced a very pronounced effect in three-dimensional culture. Specifically, MCF-7/IGF-IR cells cultured on Matrigel formed large spheroids, while untransfected MCF-7 cells, or MCF-7 cells transfected with an empty vector, aggregated in small clusters [29] (Figure 1). Moreover, the extent of cell-cell adhesion (size of spheroids) corresponded to the level of IGF-IR overexpression. Most importantly, mitogenic capacity and

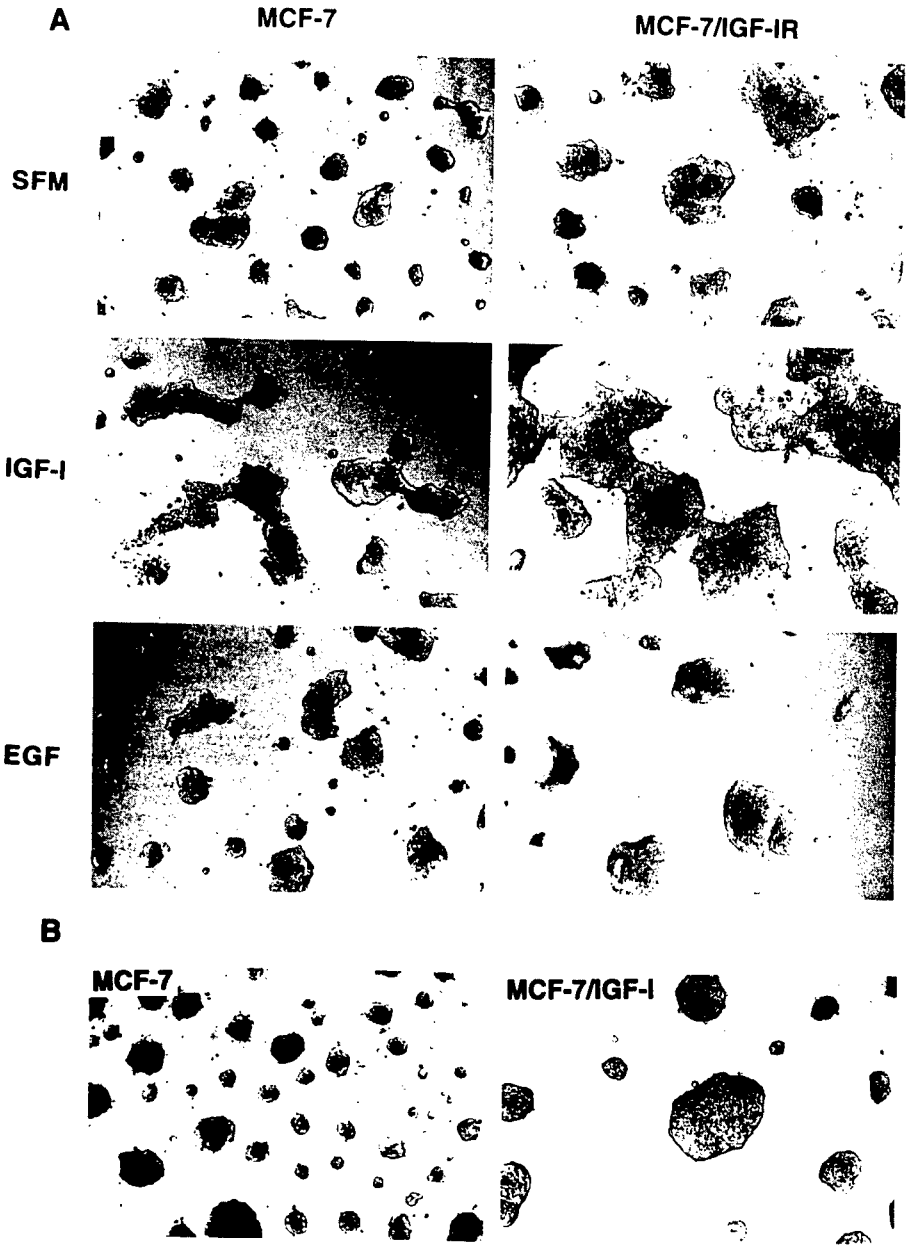

Figure 1. Activation of the IGF-IR promotes cell aggregation. **A.** Effect of IGF-I and EGF on aggregation in MCF-7 and MCF-7/IGF-IR cells. The cells (as single cell suspensions) were plated on Matrigel in a 12-well plate at a concentration of $2x10^4$/well, and cultured in PRF-SFM (control), or PRF-SFM containing 50 ng/ml IGF-I or 10 ng/ml EGF. Higher concentrations of EGF did not improve cell-cell aggregation in this assay. The spheroids were photographed 5 days after plating. Under PRF-SFM conditions, MCF-7 cells express ~ $6x10^4$ IGF-IR/cell, and MCF-7/IGF-IR, C-17 cells express $1x10^6$ IGF-IR/cell [29]. **B.** Effect of IGF-I overexpression on aggregation in MCF-7 cells. MCF-7/IGF-I cells are MCF-7 cells stably transfected with the plasmid CVN-IGF-I containing IGF-I cDNA cloned in the CVN expression vector [Nicosia and Surmacz, unpublished data]. MCF-7/IGF-I cells express immunoreactive IGF-I and their monolayer growth in PRF-SFM is enhanced ~ 4-fold compared with that of MCF-7 cells. MCF-7 and MCF-7/IGF-I cells were plated on Matrigel as described above, and cultured in DMEM:F12 containing 5% calf serum. The spheroids were photographed 10 days after plating.

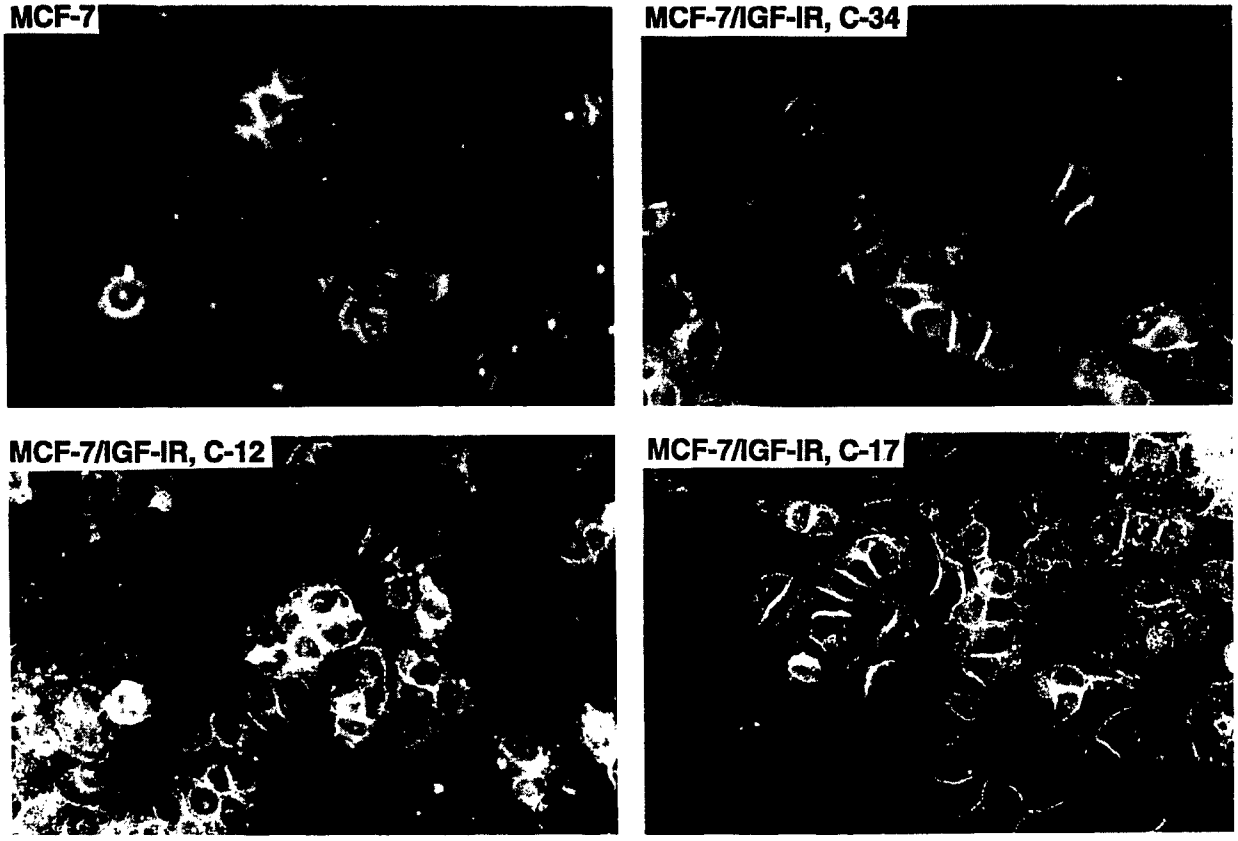

Figure 2. Localization of the IGF-IR in cell-cell contacts in MCF-7/IGF-IR cells. MCF-7 cells and MCF-7-derived clones expressing different levels of the IGF-IR were plated on coverslips in DMEM:F12 containing 5% calf serum, grown till 80% confluent, then fixed and processed for IGF-IR immunofluorescence, as described previously [29]. In respect to MCF-7 cells, the extent of IGF-IR overexpression is 8, 15, and 18-fold in MCF-7/IGF-IR, C-12, MCF-7/IGF-IR, C-34, and MCF-7/IGF-IR, C-17, respectively [29]. The fluorescence staining in the nucleoli of MCF-7 cells is nonspecific.

the time of survival of cells in three-dimensional culture was greatly enhanced in the case of the clones with the highest overexpression of the IGF-IR ($>1x10^6$ sites/cell) [29]. We have also shown that in MCF-7 cells, the formation of large spheroids is stimulated by extended activation of the IGF-IR, either through continuous exposure of the cells to exogenous IGF-I or by overexpression of IGF-I cDNA (Figure 1). In contrast, the treatment of MCF-7 cells with EGF (Figure 1), insulin, or IGF-II (Guvakova & Surmacz, unpublished data) was much less effective in promoting cell aggregation.

A principal cell-cell adhesion molecule in MCF-7 cells is E-cadherin. Loss of E-cadherin expression is often a characteristic of a motile and invasive phenotype in breast cancer cells [2]. In our cellular model, the IGF-IR-stimulated cell-cell adhesion was inhibited with an anti-E-cadherin antibody. A function of the IGF-IR in E-cadherin-dependent adhesion has further been suggested by the following observations: 1) E-cadherin and the IGF-IR co-localize in cell-cell contacts in MCF-7 cells ([29] and Figure 2); 2) the E-cadherin immunocomplex contains the IGF-IR, IRS-1, and SHC [29]; and 3) E-cadherin as well as alpha- and beta-catenins are present in anti-IGF-IR immunoprecipitates ([29] and Figure 3). At present, the mechanism of IGF-IR regulation of cell-cell adhesion is not clear. The catalytic

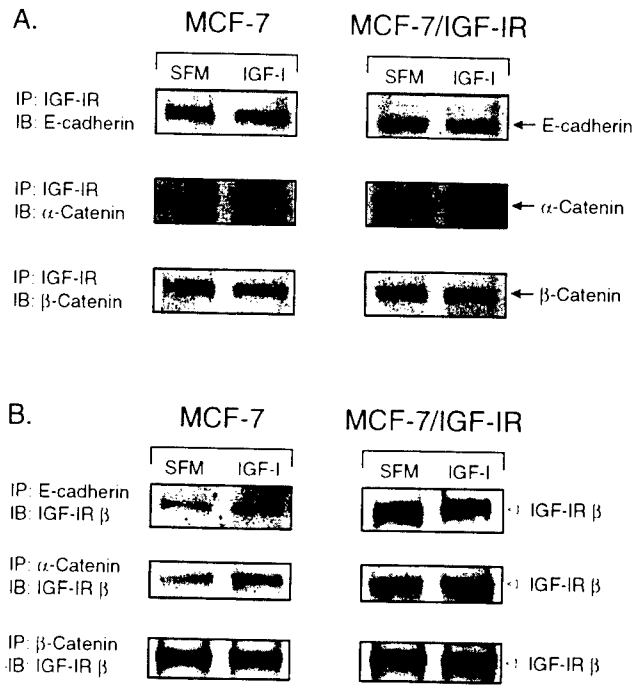

Figure 3. The IGF-IR associates with the E-cadherin complex. **A.** IGF-IR immunoprecipitates contain cell-cell adhesion proteins. 500 μg of protein lystates obtained from MCF-7 and MCF-7/IGF-IR, C-17 cells were immunoprecipitated with an anti-IGF-IR antibody. E-cadherin and α- and β-catenins were detected in the immunoprecipitates by Western blotting. The following antibodies were used: anti-IGF-IR α-IR3 (Oncogene Science), anti-E-cadherin (Transduction Laboratories), anti-α-catenin (Sigma), and anti-β-catenin (Sigma). **B.** IGF-IR associates with cell-cell adhesion proteins. 500 μg of protein lysates were immunoprecipitated with either anti-E-cadherin or anti-α or anti-β catenin antibodies. The IGF-IR was detected by Western immunoblotting with an anti-IGF-IR β subunit antibody (Santa Cruz). The antibodies used for immunoprecipitation were the same as described in A.

role of the receptor could be implied by the fact that IGF-I treatment slightly reduced tyrosine phosphorylation of a 120 kDa protein, possibly E-cadherin. It is also possible that oligomerization of receptors (due to overexpression or ligand binding) promotes their shift to cell-cell junctions. A high density of the IGF-IR in this compartment could stabilize the E-cadherin complex and strengthen adhesion. The localization of the receptors in cell-cell contacts, so prominent in the highly overexpressing clones, could restrict their availability for ligand binding and stimulation (Figure 2). Thus, the interactions between the IGF-IR and adhesion complexes may represent a mechanism by which the balance between mitogenicity and differentiation is maintained in epithelial cells.

The involvement of the IGF-IR in the stimulation of cell-cell adhesion suggests that this receptor may have anti-scattering functions. Indeed, Bracke et al. described IGF-IR-dependent increased aggregation and decreased invasive properties of MCF-7/6 cells *in vivo* [45]. On the other hand, the IGF-IR has been shown to mediate motility in breast cancer cells through the activation of integrin signaling *in vitro* [30]. In MCF-7/IGF-IR cells IGF-I-mediated motility *in vitro* was comparable to that in MCF-7 cells [Guvakova and Surmacz, unpublished data]. However, IGF-I-dependent migration was moderately reduced (20%) in cells with impaired IGF-IR signaling (MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells) ([42] and Table 2). Undoubtedly, microenvironment must play a critical role in balancing IGF-I-regulated cell-cell adhesion and cell migration.

IGF-IR signaling molecules in breast cancer cells

Functions of IRS-1 and SHC

The role of different signaling pathways and substrates in the etiology and progression of breast cancer is not clear. IRS-1 appears to be overexpressed in tumors characterized by a shorter DFS [46]. The status of SHC or GRB2 in breast tumors is not known. GRB7, an adapter of SHC, is overexpressed and co-amplified with erbB-2 [47]. In breast cancer cell lines, an increased expression of GRB2 has been noted [48], whereas SHC levels are similar to those in normal breast cells [49].

To study the function of IGF-IR signaling elements, we generated, by an antisense RNA strategy, several MCF-7-derived clones with reduced levels of either IRS-1 or SHC (MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells) [42] as well as clones overexpressing different levels of IRS-1 (MCF-7/IRS-1 cells) [34].

Down-regulation of IRS-expression by 70% resulted in the inhibition of growth in 5% CS by 60%, and cell death in PRF-SFM or PRF-SFM supplemented with IGF-I. Moreover, anchorage-independent growth of MCF-7/anti-IRS-1 cells cultured in 10%FBS was reduced by at least 70% [42]. On the other hand, the amplification of IRS-1 in MCF-7 cells reduced estrogen growth requirements, promoted autocrine growth in PRF-SFM [34], and prolonged cell survival in three-dimensional culture [Guvakova and Surmacz, unpublished data].

The studies on the mechanism of antiestrogen action confirmed an important role of IRS-1 signaling in breast cancer cell proliferation [50, 64] (Table 2). In particular, we have shown that in MCF-7, MCF-7/IGF-IR, and MCF-7/IRS-1 cells the inhibition of growth resulting from a 4 day tamoxifen treatment was accompanied by a great (~ 90%) reduction of IRS-1 tyrosine phosphorylation, without modification of IRS-1 protein levels. Under the same treatment, SHC tyrosine phosphorylation was either not affected or enhanced [64].

In MCF-7/anti-SHC cells, down-regulation of SHC levels by approximately 50% restricted monolayer growth of MCF-7 cells by ~30% in 5% calf serum, and by ~70% in PRF-SFM or PRF-SFM with 20 ng/ml IGF-I, and inhibited the growth in soft agar by 70% [42]. Notably, MCF-7/anti-SHC cells exhibited impaired ability to form spheroids in three-dimensional culture ([42] and Table 2). Thus, the IRS-1 pathway appears to regulate breast cancer cell proliferation and may be involved in cell survival. The SHC pathway also supports growth, and may play a role in the processes of cell motility and cell-cell aggregation.

Regulation of the IGF-IR by estrogens and antiestrogens

IGF-IR /ER cross-talk

In most ER^+ breast cancer cells, estrogens act in synergy with IGFs to stimulate cell proliferation [2,3,51]. In part, the effect of these steroids is mediated via sensitization of cells to IGF action. Specifically, E2 treatment up-regulates IGF-IR mRNA levels and the number of IGF-I binding sites by 2-10-fold [34,52]. On the other hand, exposure of cells to antiestrogens down-regulates IGF-IR-mediated growth [53-56]. Several studies have demonstrated decreased IGF binding in cells cultured in the presence of different antiestrogens. In particular, a 4-day tamoxifen treatment reduced the number of IGF-I binding sites in MCF-7 cells by 20-30% [55]. Similarly, 4-hydroxytamoxifen decreased IGF-I binding by 60% and 30% in MCF-7 and T47D cells, respectively [54]. Another antiestrogen, droloxifene, inhibited IGF binding in MCF-7 cells by 30-50% [55]. However, wherever the effect of antiestrogen on the IGF-IR is considered, these reports have to be taken with caution since in epithelial cells, IGF-I is known to bind not only to high affinity receptors but also to membrane IGF-IBPs (whose expression is modulated by steroids and their

antagonists) [7]. We measured the effect of tamoxifen on IGF-IR levels by Western blotting in MCF-7 and MCF-7/IGF-IR cells. A long-term treatment with 10 nM tamoxifen (4 days) reduced cell growth by at least 50%, without apparent reduction of IGF-IR protein levels; however, in MCF-7/IGF-IR cells reduced IGF-IR tyrosine phosphorylation was detectable [64]. The mechanism of tamoxifen interaction with growth factor signaling in breast cancer cells has yet to be defined, but our studies as well as the work of others [57] suggest that this antiestrogen may modulate the activity of phosphatases.

Because estrogens up-regulate the IGF-IR, it has been postulated that amplification of IGF-IR signaling may lead to estrogen-independence [52]. Indeed, MCF-7/IGF-IR and MCF-7/IRS-1 cells have reduced E2 requirements, and a maximal growth effect in these cells can be achieved with IGF-I alone [29,34]. In agreement with these findings, estrogen-independence of several *in vitro*-selected cell lines has been shown to correlate with IGF-IR overexpression [58]. However, the amplification of IGF-IR signaling and resulting estrogen-independence do not induce the ER^- phenotype. For instance, MCF-7/IRS-1 or MCF-7/IGF-IR cells retain ER expression and their growth is still regulated by the ER system [29,34,64]. Similarly, in breast tumors, the IGF-IR, even if highly overexpressed, is co-expressed with the ER ([14] and Table 1), and the occurrence of the $ER^-/IGF\text{-}IR^+$ phenotype is rare [14].

Although the molecular mechanism of ER/IGF-IR cross-talk is still not well understood, recent studies have demonstrated an interaction between signal transduction pathways of the two systems. For instance, in MCF-7 cells, one of the acute effects of E2 treatment (within 15 sec of stimulation) is src-dependent tyrosine phosphorylation of SHC and subsequent activation of the MAP kinase cascade [58]. In addition, a converse mechanism has also been described; IGF-I stimulates tyrosine phosphorylation of the ER, probably through the Ras/MAP kinase pathway, and may affect ER activity [59,60].

Regulation of the IGF-IR by IGF-II and p53

Frequently breast tumors, especially the stromal components, express IGF-II [2,36]. In cellular models, the IGF-IR mRNA and protein levels are inhibited by IGF-II, and by some factors inducing secretion of IGF-II such as progesterone [61]. Whether a similar mechanism regulates IGF-IR expression in breast tumors has not yet been determined.

The tumor suppressor protein p53 is often mutated and overexpressed in breast cancer cells, and these alterations have been associated with a worse prognosis. Interestingly, p53 appears to be co-expressed with the IGF-IR [15]. Since an inactive p53 derepresses the IGF-IR promoter [62], it has been speculated that in breast cancer, p53 may up-regulate the levels of the IGF-IR.

Concluding Remarks

The mechanism of IGF-IR overexpression in breast cancer is still not clear, but it appears that multiple factors, such as estrogens or mutant p53, may contribute to this alteration by stimulating IGF-IR transcription. Overexpressed IGF-IRs could promote the development of breast tumors by 1) sensitizing cells to low concentrations of IGFs, and 2) establishing strong cell-cell interactions. Sensitization to mitogenic action of IGFs would provide the cells overexpressing IGF-IRs with a growth advantage. Stimulation of intercellular contacts, and resulting prolonged survival of tumor cells in three-dimensional culture, could promote recurrence and/or emergence of subpopulations with a more aggressive phenotype. These functions of the IGF-IR should be considered in designing anti-IGF-IR modalities for breast cancer treatment. In addition, targeting specific substrates of the IGF-IR such as IRS-1, which is involved in the control of survival and proliferation, could prove especially beneficial. Moreover, the functional relations between the IGF-IR and the ER indicate that endocrine therapy in breast cancer should aim at both systems.

However, before such molecular therapies are in effect, further studies are needed to better define the role of the IGF-IR in breast disease. For instance, the role of the IGF-IR in malignant progression of breast cancer is still obscure, especially considering that a low IGF-IR content has been correlated with high risk breast cancer. Is this phenomenon related to the fact that more aggressive tumors usually express high levels of IGF-II, which, in turn, may down-regulate IGF-IR expression? Is, in this milieu, IGF-II acting through the IGF-IR, or is another receptor involved? Could down-regulation of IGF-IRs lead to the development of a more motile and invasive phenotype in breast cancer? Is E-cadherin expression correlated with IGF-IR status in breast tumors? The research efforts in our and other laboratories are directed towards answering these questions.

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Advances in Brief

Tamoxifen Interferes with the Insulin-like Growth Factor I Receptor (IGF-IR) Signaling Pathway in Breast Cancer Cells[1]

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Abstract

The insulin-like growth factor I receptor (IGF-IR) is involved in the control of breast cancer cell growth. The cytostatic activity of tamoxifen (Tam), a nonsteroidal antiestrogen, is partially mediated through interference with IGF-I-R-dependent proliferation, yet the effects of Tam on IGF-IR intracellular signaling have never been elucidated. Consequently, we investigated how Tam modifies the IGF-IR signaling pathway in estrogen receptor-positive MCF-7 breast cancer cells and in MCF-7-derived clones overexpressing either the IGF-IR (MCF-7/IGF-IR cells) or its major substrate, IRS-1 (MCF-7/IRS-1 cells). MCF-7/IGF-IR and MCF-7/IRS-1 cells exhibit greatly reduced estrogen growth requirements but retain estrogen receptors and express sensitivity to antiestrogens comparable to that in the parental cells. In all tested cell lines, regardless of the amplification of IGF signaling, a 4-day treatment with 10 nM Tam produced a similar cytostatic effect. In MCF-7 and MCF-7/IGF-IR cells, growth inhibition by Tam was associated with the reduced tyrosine phosphorylation of the IGF-IR in the presence of IGF-I; however, the basal level of the IGF-IR remained unaffected. Moreover, Tam inhibited both basal and IGF-I-induced tyrosine phosphorylation of IRS-1, which was accompanied by down-regulation of IRS-1-associated phosphatidylinositol 3′-kinase activity and reduced IRS-1/growth factor receptor-bound protein 2 (GRB2) binding. In contrast, under the same treatment, tyrosine phosphorylation of Src-homology/collagen proteins (SHC; another substrate of the IGF-IR) and SHC/GRB2 binding were elevated. The protein levels of the IGF-IR and IRS-1 were not modified by Tam, whereas SHC protein expression was either not affected or moderately decreased by the treatment.

In summary, this work provides the first evidence that in MCF-7 cells, cytostatic effects of Tam are associated with the modulation of IGF-IR signaling, specifically with: (*a*) down-regulation of IGF-I-induced tyrosine phosphorylation of the IGF-IR; (*b*) inhibition of IRS-1/phosphatidylinositol 3′-kinase signaling; and (*c*) up-regulation of SHC tyrosine phosphorylation and increased SHC/GRB2 binding. It is hypothesized that dephosphorylation of IRS-1 could be a major contributing factor in Tam cytostatic activity.

Introduction

The activation of the IGF-IR,[3] through a paracrine, autocrine, or endocrine mechanism, appears to play a critical role in the regulation of breast cancer cell growth (1). The IGF-IR levels are significantly higher in breast cancer than in normal breast tissue or benign tumors (1–3). The IGFs are potent mitogens for cultured breast cancer cells, and their expression has been documented in the epithelial and/or stromal component of breast tumors (1). In primary breast cancer, a correlation has been found between tumor size, the levels of IRS-1 (a cellular substrate of the IGF-IR), and recurrence of the disease (4). In MCF-7 breast cancer cells, the overexpression of either IRS-1 (5), the IGF-IR (6), or IGF-II (7) have been shown to reduce estrogen growth dependence. On the other hand, it has been demonstrated that blockade of IGF-IR signaling with, for instance, anti-IGF-IR antibodies (1), antisense RNA to the IGF-IR (8), and antisense oligodeoxynucleotides to IRS-1 (5) restricts breast cancer cell growth *in vitro* or *in vivo*.

The activation of the IGF-IR tyrosine kinase results in the stimulation of diverse intracellular pathways involving different signaling substrates (9). The best characterized substrates of the IGF-IR are IRS-1 and SHC. IRS-1 is a docking protein that, upon tyrosine phosphorylation by the IGF-IR, recruits several effector proteins through SH2-type interactions. For instance, IRS-1 binds and activates PI-3 kinase and SYP phosphatase as well as stimulates Ras/MAP pathway through the binding of GRB-2/SOS complexes (9). Moreover, IRS-1 has been found to interconnect with JAK-STAT (10) and integrin signaling pathways (11). SHC proteins are substrates of most tyrosine kinase receptors, many nonreceptor kinases and certain phosphatases (12, 13). Tyrosine phosphorylated SHC, similar to IRS-1, may activate Ras/MAP signaling cascade through the GRB2/SOS complex (12).

Tam, a nonsteroidal antiestrogen with partial agonist activity, is commonly used in adjuvant therapy in breast cancer management (14). Tam inhibits ER-dependent growth but also interferes with polypeptide growth factor signaling (14). The known effects of Tam or its derivative 4-OH-Tam on the IGF system in breast cancer cells include: inhibition of IGF-I stimulated growth (14, 15), modulation of IGFBP expression (1), reduced secretion of autocrine IGF (16), down-regulation of plasma levels of IGF-I in breast cancer patients (17), and decreased levels of IGF-I binding sites (18, 19). The interaction of Tam with the IGF-IR signaling pathway has not been characterized, partly because of the lack of adequate cellular models. Here, we investigated this aspect of Tam action using ER-positive MCF-7 cells as well as different MCF-7-derived cell lines overexpressing the elements of IGF-IR signaling.

Materials and Methods

Cell Lines and Cell Culture Conditions. MCF-7 cells were routinely grown in DMEM:F12 (1:1) containing 5% calf serum (6). In the experiments requiring estrogen-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM $FeSO_4$ and 2 mM L-glutamine (PRF-SFM; Ref. 6).

MCF-7/IGF-IR, clones 12 and 15, and MCF-7/IRS-1, clone 3 were developed by stable transfection with the expression vectors pcDNA3/IGF-IR and CMV-IRS-1, respectively, and were characterized in detail previously (5, 6). The clones were maintained in culture for a maximum of 3 months in growth medium supplemented with 200 μg/ml G418.

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[3] The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; ER, estrogen receptor; GRB2, growth factor receptor-bound protein 2; IGFBP, IGF binding protein; IRS-1, insulin receptor substrate 1; MCF-7/IGF-IR, MCF-7 cells overexpressing IGF-IRs; MCF-7/IRS-1, MCF-7 cells overexpressing IRS-1; PI-3 kinase, phosphatidylinositol 3′-kinase; PRF-SFM, phenol red-free serum-free medium; SH2, src homology 2 domain; Tam, tamoxifen; SHC, Src-homology/collagen proteins; MAP, mitogen-activated protein kinase.

Cell Growth Assay. Cells (1×10^5) were plated in 24-well plates in DMEM:F12 (1:1) containing 5% calf serum. The next day, designed as day 0 of the experiment, the cells were shifted to either PRF-SFM or PRF-SFM supplemented with 0.1–100 nM Tam. For each cell line, the number of cells at day 0 was taken as 100% (control). The relative increase (percentage over control) in cell number was determined after 4 days of Tam treatment.

Western Blotting and Immunoprecipitation. The levels of the IGF-IR, IRS-1, SHC, as well as tyrosine phosphorylation of these proteins, were measured by Western blotting. The protein lysates (250–500 μg) were obtained as described previously (6) and immunoprecipitated with the following antibodies: for IGF-IR, anti-IGF-IR monoclonal antibody alpha-IR3 (Oncogene Science); for IRS-1, anti-IRS-1 polyclonal antibody (UBI); and for SHC, anti-SHC polyclonal antibody (Transduction Laboratories). The immunoprecipitates were resolved by PAGE, and the IGF-IR, IRS-1, or SHC proteins were immunodetected with the following antibodies: for IRS-1, anti-IRS-1 polyclonal antibody (UBI); for IGF-IR and its M_r 200,000 precursor (20), anti-IGF-IR polyclonal antibody (Santa Cruz); and for SHC, anti-SHC monoclonal antibody (Transduction Laboratories). Tyrosine phosphorylation of the above proteins was detected by immunoblotting with an anti-phosphotyrosine monoclonal antibody PY20 (Transduction Laboratories). The intensity of bands was assessed by laser densitometry scanning.

PI-3 Kinase Activity. The activity of PI-3 kinase associated with IRS-1 was assessed by standard protocol provided by the manufacturer of the IRS-1 antibody (UBI). In brief, 500 μg of protein lysate were immunoprecipitated with an anti-IRS-1 polyclonal antibody. The IRS-1 immunoprecipitates were incubated *in vitro* in the presence of 200 μg/ml phosphatidylinositol (Sigma Chemical Co.) and 10 μCi[γ-^{32}P]ATP for 30 min. The products of the kinase reaction were resolved on TLC plates (Eastman Kodak), and the spots corresponding to PI-3 phosphates were identified by autoradiography. The spots were then cut from the plates, and their radioactivity was counted with a beta counter. For each cell line, PI-3 kinase activity obtained in SFM was taken as 100% (control).

Results

Tam Inhibits the Growth of MCF-7 Breast Cancer Cells Overexpressing Either the IGF-IR or IRS-1. We studied whether Tam is able to inhibit the growth of MCF-7 cells with amplified IGF-IR signaling (MCF-7/IGF-IR and MCF-7/IRS-1 cells). The estrogen growth requirements in these cells are abolished or significantly reduced; however, the cells retain expression of the ER (5, 6). The effect of Tam on growth was studied in MCF-7/IGF-IR, clone 12 (expressing 5×10^5 IGF-I binding sites/cells: 8-fold IGF-IR overexpression over the levels in MCF-7 cells), MCF-7/IGF-IR, clone 15 (3×10^6 sites/cells: 50-fold IGF-IR overexpression), and in MCF-7/IRS-1, clone 3 (a 9-fold overexpression of IRS-1 over that in MCF-7 cells); MCF-7 cells were used as a control. The cells were cultured in PRF-SFM for 4 days in the presence of different concentrations of Tam (0.1–100 nM). Tam treatment suppressed growth of all cell lines in a dose-dependent manner (Fig. 1). Specifically, in all cells, 0.1, 1.0, and 10 nM Tam reduced proliferation by at least 12, 34, and 50%, respectively. The extent of Tam-induced growth inhibition in cells cultured for 4 days in PRF-SFM with 20 ng/ml IGF-I was comparable (data not shown). Treatment with 100 nM Tam was always cytotoxic. Consequently, Tam at a concentration of 10 nM was used in all further experiments.

Tam Interferes with IGF-I-induced Tyrosine Phosphorylation of the IGF-IR in MCF-7/IGF-IR Cells. To investigate the effects of Tam on IGF-IR signaling, we assessed tyrosine phosphorylation and protein levels of the IGF-IR in MCF-7 and MCF-7/IGF-IR, clone 15 cells. In cells cultured in PRF-SFM plus IGF-I, the IGF-IR tyrosine phosphorylation was always elevated compared with that in PRF-SFM (Fig. 2). After 4 days of treatment, the effects of Tam on the basal level of IGF-IR tyrosine phosphorylation were minimal (Fig. 2*A*); specifically, in several experiments either no modification or slight (~15%) up- or down-regulation were noticeable. However, Tam reduced IGF-I-induced tyrosine phosphorylation by 60% in MCF-7 cells and by 30% in MCF-7/IGF-IR cells (Fig. 2*B*).

The IGF-IR protein levels were not significantly modulated by Tam, as determined by laser densitometry scanning (Fig. 2). Similarly, the levels of the IGF-IR precursor were not affected by the treatment (Fig. 2*A*).

Inhibition of Cell Growth by Tamoxifen Is Associated with Dephosphorylation of IRS-1. In all tested cell lines, but especially in the clones with amplified IGF-IR signaling (MCF-7/IGF-IR, clones 12 and 15, and in MCF-7/IRS-1 cells), a basal level of IRS-1 tyrosine phosphorylation was evident even after prolonged culture in PRF-SFM, which reflected cellular response to autocrine IGFs, as shown previously (Refs. 5 and 6; Fig. 3, *A* and *B*). The addition of 10 nM Tam to PRF-SFM produced a cytostatic effect (Fig. 1), which was accompanied by a marked dephosphorylation of IRS-1 on tyrosine residues in the cells studied. Specifically, the basal level of IRS-1 tyrosine phosphorylation was reduced by 29, 35, and 48% in MCF-7/IGF-IR, clone 12, MCF-7/IGF-IR, clone 15, and MCF-7/IRS-cells, respectively (Fig. 3*A*). In MCF-7 cells, due to a low basal level of IRS-1

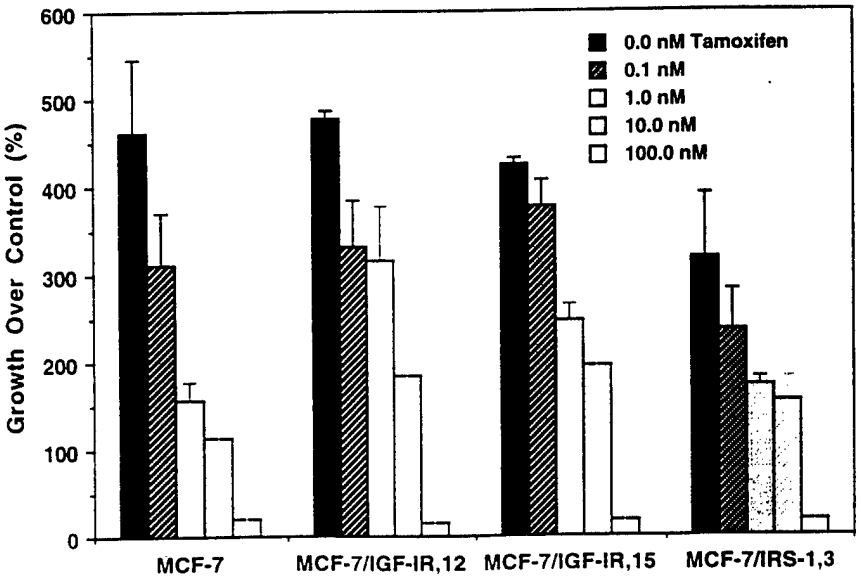

Fig. 1. Tam inhibits the growth of MCF-7 cells overexpressing either the IGF-IR or IRS-1. The cells were treated as described in "Materials and Methods." The results represent the percentage of growth inhibition relative to control (100%) in PRF-SFM. The results are means from at least four experiments. *Bars*, SE.

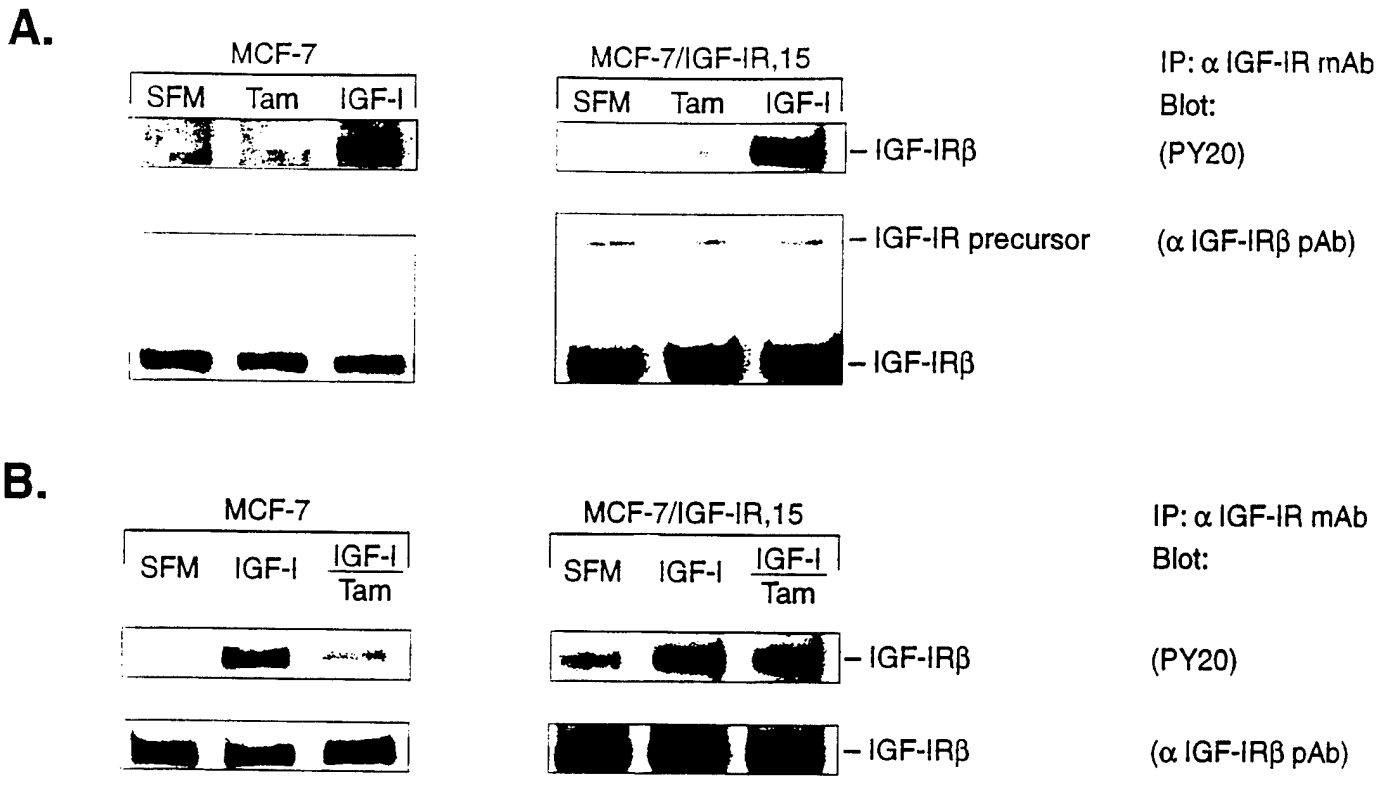

Fig. 2. Effects of Tam on the IGF-IR. *A*, Tam effect on basal level of IGF-IR tyrosine phosphorylation. The cells were lysed after 3 days of incubation in either PRF-SFM (*SFM*), PRF-SFM plus 10 nM Tam (*Tam*), or PRF-SFM plus 50 ng/ml IGF-I (*IGF-I*). Tyrosine phosphorylation and protein level of the IGF-IR were determined after immunoprecipitation of 500 μg of protein lysates with an anti-IGF-IR monoclonal antibody followed by Western blotting with the indicated antibodies. *B*, Tam blocks IGF-I-induced tyrosine phosphorylation of the IGF-IR. *Lane IGF-I/Tam*, cells were cultured in PRF-SFM with 50 ng/ml IGF-I and 10 nM Tam; other conditions were as described for *A*. Representatives results from four experiments are shown.

phosphorylation, the effect of Tam was not measurable. The interference of Tam with IRS-1 signaling was further studied in MCF-7/IRS-1 cells (Fig. 3*B*). The IGF-I-stimulated and basal levels of IRS-1 phosphorylation were suppressed in the presence of the drug by approximately 43% (Fig. 3*B*). The dephosphorylation of IRS-1 was accompanied by its dissociation from both p85 subunit of PI-3 kinase and GRB2 (Fig. 3*B*). Similar effects of Tam on IRS-1 tyrosine phosphorylation (approximately 27% inhibition) were seen in MCF-7/IGF-IR, clone 15 cells (data not shown).

In addition, Tam suppressed the activity of IRS-1-associated PI-3 kinase in cells stimulated with IGF-I; the inhibition by 43, 92, and 128% was seen in MCF-7, MCF-7/IGF-IR, and MCF-7/IRS-1, respectively (Fig. 3*C*). The effects of Tam on PI-3 kinase in cells cultured in PRF-SFM were not measurable.

In several repeat experiments, IRS-1 protein levels were not affected by long-term treatment with Tam (Fig. 3, *A* and *B*).

Tamoxifen Increases Tyrosine Phosphorylation of SHC. Of note, in all studied cell lines the cytostatic action of Tam was associated with the elevated tyrosine phosphorylation of $p52^{SHC}$ and $p46^{SHC}$ (Fig. 4). The activation of $p52^{SHC}$ was especially prominent; specifically, compared with SHC status in untreated cells, a 34, 110, and 100% augmentation of $p52^{SHC}$ tyrosine phosphorylation was observed in MCF-7, MCF-7/IGF-IR, and MCF-7/IRS-1 cells, respectively. Moreover, the hyperphosphorylation of $p52^{SHC}$ was followed by an its increased binding to GRB2 (Fig. 4).

In contrast, in all cell lines, a 4-day exposure to IGF-I decreased tyrosine phosphorylation of $p52^{SHC}$ by approximately 40% compared with that in PRF-SFM and induced dissociation of SHC/GRB2 complexes (Fig. 4).

Tam treatment produced a consistent down-regulation of $p52^{SHC}$ and $p46^{SHC}$ levels by approximately 35% in MCF-7/IGF-IR, clone 15 and MCF-7/IRS-1 cells but not in MCF-7 cells (Fig. 4). In contrast, SHC protein expression was not modulated by IGF-I (Fig. 4).

Effect of Tamoxifen on ERK2. Because IRS-1 and SHC, via GRB2/SOS, may activate Ras/MAP signaling pathway, we assessed MAP (ERK2) kinase activity in cells exposed to Tam or cultured for 4 days in PRF-SFM in the presence or absence of exogenous IGF-I. We found no differences in ERK2 activity under these conditions, measured in an *in vitro* assay, using myelin basic protein as a substrate (data not shown).

Discussion

Experimental evidence suggests an important role of the IGF-R in the pathobiology of breast tumors (1–3). Activation of the IGF-IR promotes proliferation and transformation as well as cell-cell and cell-substrate interactions in breast cancer cells (1, 5, 6, 21). Conversely, the blockade of IGF signaling results in the inhibition of breast cancer growth (1, 5, 8). Tam, or its derivative 4-OH-Tam, have been shown to inhibit IGF-IR-dependent growth through different mechanisms, such as down-regulation of autocrine IGF secretion (16) or modulation of IGFBPs expression (1). In addition, in MCF-7 breast cancer cells, Tam and 4-OH-Tam decreased expression of IGF-I binding sites by approximately 30% (19) and 60% (18), respectively.

The effects of Tam on the IGF signal transduction pathway are unknown. Here, we report for the first time the modulation of the IGF-IR intracellular signaling pathways associated with the cytostatic action of Tam. Our studies focused on tyrosine kinase activity and protein levels of the IGF-IR and its two major cellular substrates, IRS-1 and SHC. Preliminary data from Rocha *et al.* (4) documented that IRS-1 is expressed in primary breast tumors and its levels correlate with increased recurrence. The status of SHC and its relation with

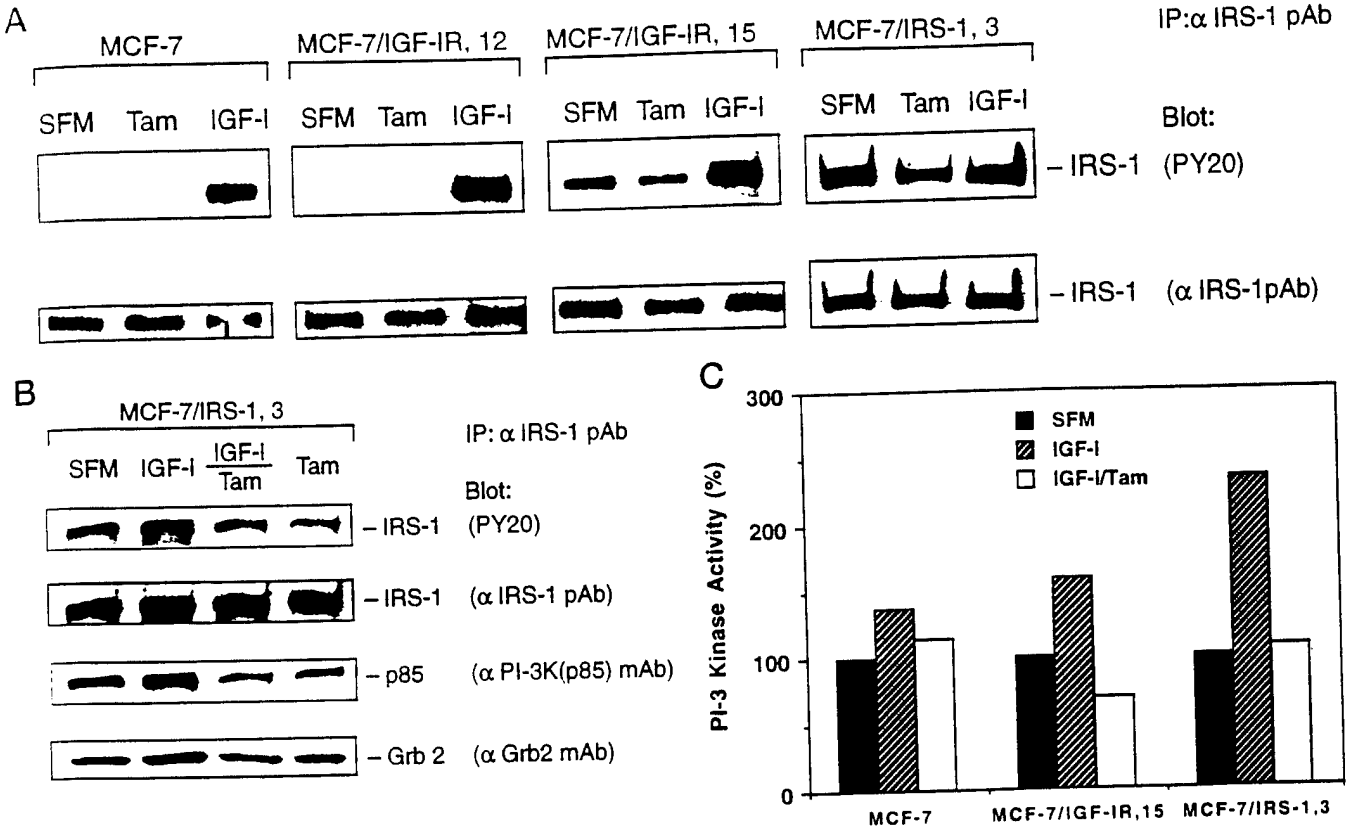

Fig. 3. Effects of Tam on IRS-1-mediated signaling. *A*, effects of Tam on IRS-1 tyrosine phosphorylation. MCF-7 cells, MCF-7/IGF-IR, clones 12 and 15, and MCF-7/IRS-1, clone 3, were incubated in PRF-SFM (*SFM*), PRF-SFM plus 10 nM Tam (*Tam*), or PRF-SFM plus 50 ng/ml IGF-I (*IGF-I*) for 3 days. IRS-1 was immunoprecipitated from 300 μg of protein lysates, and tyrosine phosphorylation levels were detected with PY-20 antibody. IRS-1 protein level was determined in the original blot, after stripping and reprobing with an anti-IRS-1 antibody. Representative results from five experiments are shown. *B*, effects of Tam on IRS-1 signaling in MCF-7/IRS-1 cells. MCF-7/IRS-1, clone 3, was cultured for 4 days in either PRF-SFM (*SFM*), PRF-SFM plus 50 ng/ml IGF-I alone (*IGF-I*), PRF-SFM plus 50 ng/ml IGF-I with 10 nM Tam (*IGF-I/Tam*), or PRF-SFM plus 10 nM Tam (*Tam*). IRS-1 was immunoprecipitated from 250 μg of lysates, and IRS-1 protein level and tyrosine phosphorylation were detected as described above. Amounts of p85 of PI-3 kinase and GRB2 associated with IRS were determined in original nitrocellulose filters after stripping and reprobing with specific antibodies. *C*, Tam effects on IRS-1 associated PI-3 kinase activity. The cells were incubated in PRF-SFM (*SFM*), PRF-SFM plus 50 ng/ml IGF-I (*IGF-I*), or PRF-SFM plus 50 ng/ml IGF-I and 10 nM Tam (IGF-I/Tam) for 3 days. IRS-1 was precipitated from 500 μg of cell lysates from each cell line. The activity of PI-3 kinase associated with IRS-1 was assessed *in vitro* as described in "Materials and Methods." The results are expressed as percentage of increase over control levels in SFM (100%). Representative data are shown.

ifferent prognostic markers is not known. Our experiments with ICF-7 cells expressing antisense RNA to either IRS-1 or SHC demonstrated that normal levels of both substrates are critical in sustaining monolayer and anchorage-independent growth.[4] In addition, IRS-1 signaling appears to play a role in the protection from apoptosis *in vitro*.[4]

Here, we looked at the status of IRS-1 and SHC in the state of growth inhibition induced by Tam. We approached this problem using MCF-7 cells as well as more sensitive cellular models, *i.e.*, MCF-7-derived clones overexpressing either the IGF-IR or IRS-1 (5, 6). The amplification of IGF-IR signaling in MCF-7 cells promotes growth responsiveness to IGF-I and abrogates estrogen growth requirements but does not influence ER expression and function (5, 6). In this work, we demonstrated that estrogen independence in MCF-7/IGF-IR and MCF-7/IRS-1 cells does not circumvent sensitivity to cytostatic action of Tam. Remarkably, the inhibition of growth was similar in all studied cell lines, regardless of the amplification of IGF-IR signaling. With this experimental system we have made several important observations:

(*a*) Tam had no apparent effect on IGF-IR protein levels or the levels of its precursor, at least in a 4-day experiment. Other studies demonstrated a small (30%) down-regulation of IGF binding sites in Tam-treated MCF-7 cells (19); however, binding assays were performed without discriminating IGF-I association with membrane IGFBPs, which could result in miscalculation of IGF-IR levels (22).

(*b*) Tam inhibited IGF-I-induced tyrosine phosphorylation of the IGF-IR. It is unlikely that this effect was mediated exclusively through the reduction of the amount of autocrine IGFs because Tam effectively suppressed autocrine growth without modification of the basal IGF-IR tyrosine phosphorylation (in PRF-SFM; Figs. 1 and 2). Why the effect of Tam on IGF-IR activation is evident in the presence of excess IGF-I but not with autocrine IGFs remains to be clarified; possibly, the regulation of the phosphatase system is different under these two conditions. This observation, however, suggests that continuing dephosphorylation of the IGF-IR is not critical for Tam-induced growth arrest.

(*c*) Tam treatment resulted in the persisting dephosphorylation of IRS-1 on tyrosine residues, apparently in the presence of both autocrine and exogenous IGF-I. The attenuation of IRS-1 tyrosine phosphorylation by Tam was accompanied by down-regulation of IRS-1-associated PI-3 kinase activity and dissociation of GRB2 form IRS-1. Our findings agree with preliminary data of Kleinman *et al.* (23), who demonstrated that Tam inhibited tyrosine phosphorylation of a M_r 185,000 protein (possibly IRS-1) in MCF-7 cells.

(*d*) The effect of Tam on SHC was evidently different from that seen

[4] M. Nolan, L. Jankowska, M. Prisco, S. Xu, M. Guvakova, and E. Surmacz. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. Int. J. Cancer, in press.

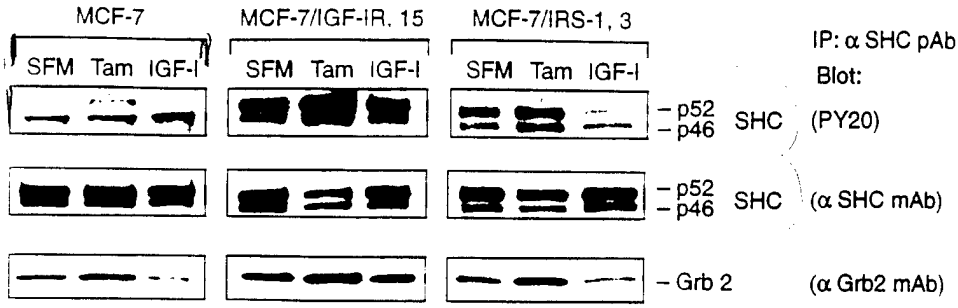

Fig. 4. Effects of Tam on SHC signaling. The cells were grown in either PRF-SFM (*SFM*), PRF-SFM plus 10 nM Tam (*Tam*), or PRF-SFM plus 50 ng/ml IGF-I (*IGF-I*) for 3 days. SHC protein were immunoprecipitated from 500 μg of cell lysates with an anti-SHC polyclonal antibody followed by detection of tyrosine phosphorylation of SHC with PY-20. The SHC protein and SHC-associated GRB2 were detected in original filters, upon stripping and re-probing with specific antibodies. Representative results of five experiments are shown.

for IRS-1. Here, growth inhibition was associated with elevated tyrosine phosphorylation of SHC proteins, especially $p52^{SHC}$, without up-regulation of SHC protein levels. Importantly, long-term treatment with IGF-I, which promoted growth, concomitantly reduced SHC phosphorylation. Whether up-regulation of SHC phosphorylation is a universal feature of growth arrest or it only represents a characteristic of Tam action is presently unclear. In MCF-7 cells, treatment with genistein or herbimicin inhibited proliferation, which was associated with a reduction of SHC tyrosine phosphorylation observed after 30 min treatment (24). Longer effects of these tyrosine kinase inhibitors were not studied. In our system, higher phosphorylation of SHC in Tam-treated cells was accompanied by GRB2 binding to SHC; however, activation of ERK2 was not observed. Possibly, under Tam treatment, activation of ERK2 via SHC was counteracted by deactivation of this pathway due to disruption of IRS-1 signaling. Alternatively, as suggested by others (24, 25), the ERK2 pathway is not critical in IGF-stimulated growth in MCF-7 cells; thus, it is not a target for Tam action.

In summary, these results demonstrate that Tam differentially modulates IGF-IR signaling in breast cancer cells. The cytostatic effect of Tam is mediated by a continuing inhibition of IRS-1/PI-3 kinase pathway. On the other hand, Tam increases tyrosine phosphorylation of SHC and SHC/GRB2 binding. The biological consequences of the latter effects are presently unknown.

One possible target of Tam action is the tyrosine phosphatase system. Indeed, Freiss and Vignon (26) have recently shown that 4-hydroxytamoxifen up-regulates protein tyrosine phosphatase activity in breast cancer cells. We speculate that Tam activates, most probably through an indirect mechanism, a specific tyrosine phosphatase(s) acting upon IRS-1. On the other hand, Tam may also inhibit tyrosine phosphatase(s) that would specifically affect SHC and/or the IGF-IR. Future experiments with Tam and pure antiestrogens will further explore this issue, especially in relation with such phenomenon as antiestrogen resistance or Tam-induced growth.

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DIFFERENTIAL ROLES OF IRS-1 AND SHC SIGNALING PATHWAYS IN BREAST CANCER CELLS

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Several polypeptide growth factors stimulate breast cancer growth and may be involved in tumor progression. However, the relative importance of diverse growth factor signaling pathways in the development and maintenance of the neoplastic phenotype is largely unknown. The activation of such growth factor receptors as the insulin-like growth factor I receptor (IGF-I R), erbB-type receptors (erbB Rs) and FGF receptors (FGF Rs) controls the phenotype of a model breast cancer cell line MCF-7. To evaluate the function of 2 post-receptor signaling molecules, insulin receptor substrate-1 (IRS-1) (a major substrate of the IGF-IR) and SHC (a common substrate of tyrosine kinase receptors), we developed several MCF-7-derived cell clones in which the synthesis of either IRS-1 or SHC was blocked by antisense RNA. In MCF-7 cells, down-regulation of IRS-1 by 80–85% strongly suppressed anchorage-dependent and -independent growth and induced apoptotic cell death under growth factor- and estrogen-reduced conditions. The reduction of SHC levels by approximately 50% resulted in the inhibition of monolayer and anchorage-independent growth but did not decrease cell survival. Importantly, cell aggregation and the ability of cells to survive on the extracellular matrix were inhibited in MCF-7/anti-SHC clones, but not in MCF-7/anti-IRS-1 clones. Cell motility toward IGF was not attenuated in any of the tested cell lines, but motility toward EGF was decreased in MCF-7/anti-SHC clones. Our results suggest that in MCF-7 cells: 1) both IRS-1 and SHC are implicated in the control of monolayer and anchorage-independent growth; 2) IRS-1 is critical to support cell survival; 3) SHC is involved in EGF-dependent motility; and 4) normal levels of SHC, but not IRS-1, are necessary for the formation and maintenance of cell-cell interactions. *Int. J. Cancer* 72:828–834, 1997.
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Several polypeptide growth factors such as the insulin-like growth factors I and II (IGFs), the ligands of the erbB family of receptors (erbB Rs) and fibroblast growth factors (FGFs) regulate breast cancer growth and may be involved in breast cancer progression (Dickson and Lippman, 1995). The impact of these factors on the phenotype of breast cancer cells depends on the level and activity of their cognate membrane receptors. The growth of a model breast cancer cell line, MCF-7, is greatly stimulated by activation of the insulin-like growth factor I receptor (IGF-IR) and the epidermal growth factor receptor (EGFR) (Dickson and Lippman, 1995; Van der Burg *et al.,* 1988; Karey and Sibrascu, 1988).

The signal transduction pathways of the IGF-IR and the EGFR share several common substrates; one, for example, is SHC (Giorgetti *et al.,* 1994; Pelicci *et al.,* 1992). SHC proteins (p66, p52, p47) bind to the IGF-I or EGF receptors through a PTB or an SH2 domain (Tartare-Deckert *et al.,* 1995; Kavanaugh and Williams, 1994; Pelicci *et al.,* 1992). This association results in tyrosine phosphorylation of SHC proteins, which are then able to recruit other signaling molecules, for instance GRB-type adapters, and activate downstream signaling pathways, such as Ras/MAP kinase cascade (Giorgetti *et al.,* 1994; Skolnik *et al.,* 1993; Pelicci *et al.,* 1992).

The transmission of the IGF signal involves insulin receptor substrate 1 (IRS-1), which is not implicated in EGF signaling (Myers *et al.,* 1994; Rubin and Baserga, 1995). IRS-1 is a docking protein containing multiple tyrosine residues, which become rapidly phosphorylated upon receptor activation. This allows association of IRS-1 with different SH2-domain containing proteins and induction of various signaling pathways, such as Ras/MAP kinase (through an adapter GRB2), PI-3 kinase (through a p85 regulatory subunit) or SHPTP2 protein tyrosine phosphatase (Myers *et al.,* 1994). Ultimately, some of the signals generated by growth factors stimulate nuclear events (Myers *et al.,* 1994; Dickson and Lippman, 1995), while others are involved in the reorganization of cell morphology (Joneson *et al.,* 1996).

The significance of IRS-1- and SHC-dependent signaling in the biology of breast tumor cells is not clear. Preliminary data suggest that IRS-1 may regulate the proliferation of tumor cells. In MCF-7 cells, overexpression of IRS-1 enhanced monolayer and anchorage-independent growth and reduced growth requirements for estrogen (E2) (Surmacz and Burgaud, 1995). In primary breast tumors, a correlation has been reported between IRS-1 levels and recurrence of the disease (Rocha *et al.,* 1995). GRB2, an adapter linking IRS-1 and SHC to Ras/MAP kinase, is often overexpressed in breast cancer cell lines (Daly *et al.,* 1994). GRB7, a different adapter of SHC, is overexpressed and co-amplified with erbB2 in breast tumors (Stein *et al.,* 1994). The status of SHC proteins in breast cancer cell lines or tumor samples remains unknown.

Here we evaluated the roles of SHC and IRS-1 in growth, survival, transformation, migration toward chemo-attractants and cell-cell aggregation in MCF-7 breast cancer cells.

MATERIAL AND METHODS

Expression plasmids

To generate the sense and antisense SHC expression plasmids, a 287 bp fragment of a human SHC cDNA (from nt 55 to nt 342) was amplified by PCR using the pMJ/SHC plasmid (a kind gift of Dr. J. Schlessinger, New York, NY) as a template and oligonucleotides 5′-GTG CGG AGA CTC CAT GAG-3′ and 5′-CTC ACA CAC CAG ACT GAT G-3′, as the upstream and downstream primers, respectively. The amplified SHC DNA fragment was cloned into the pCR3 expression plasmid (Invitrogen, San Diego, CA) in either a 5′-3′ or 3′-5′ orientation to produce sense-SHC or antisense-SHC expression vectors (respectively). In the resulting expression vectors, transcription of sense or antisense-SHC RNA was driven by the CMV promoter. The expression plasmids also encoded neomycin resistance to allow for selection in G418.

The antisense- and sense-IRS-1 expression plasmids have been described previously (D'Ambrosio *et al.,* 1995). The plasmids contain the entire sequence of mouse IRS-1 cDNA cloned in either the sense or antisense direction in pRc/CMV expression vector (Invitrogen).

Cell lines and cell culture conditions

MCF-7/antisense-SHC (anti-SHC) and MCF-7/antisense-IRS-1 (anti-IRS-1) clones were generated by stable transfection using the

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calcium phosphate precipitation method. The clones were selected in 2 mg/ml G418, and the integration of transfected plasmids into genomic DNA was confirmed by PCR. In all cases, a "T7 primer" 5′-CGA CTC ACT ATA GG-3′ (located in the T7 promoter of all expression plasmids) was used as an upstream primer. The following downstream primers were used: for sense IRS-1 clones: 5′-GGC TTC TCA GAC GTG CGC AAG-3′; for antisense IRS-1 clones: 5′-GAT AAC TGC TAG GAG ACC-3′; for sense SHC clones: 5′-CTC ACA CAC CAG ACT GAT G-3′; and for antisense SHC clones: 5′-CTG CGG AGA CTC CAT GAG-3′. From each transfection, 13 PCR-positive clones were tested for the levels of target protein by Western immunoblotting (see below).

MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells were maintained in DMEM: F12 supplemented with 5% calf serum (CS) containing 200 μg/ml G418. In the experiments requiring growth factor- and estrogen-reduced conditions, we used DMEM without phenol red (PRF-DMEM) with 0.5 mg/ml BSA, 1 μM $FeSO_4$ and 2 mM L-glutamine (PRF-serum free media, PRF-SFM).

Western blotting

The reduction of SHC and IRS-1 protein levels in MCF-7 clones was confirmed by Western immunoblotting. In MCF-7/anti-IRS-1 clones, cell lysates (1.5 mg) were immunoprecipitated with an anti-IRS-1 antibody (UBI, Lake Placid, NY) and probed with another anti-IRS-1 antibody (obtained from Dr. M. Myers, Boston, MA). The same method was used to assess the levels of IRS-1 in MCF-7/anti-SHC clones, except that 500 μg of protein lysate were used for immunoprecipitation.

In MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones, SHC proteins were immunodetected with an anti-SHC monoclonal antibody (Transduction Labs, Lexington, KY) in 50 μg of total cell lysate. The levels of IRS-1 and SHC proteins were approximated by laser densitometry reading.

Anchorage-dependent growth assay

Cells were plated at a concentration of 1×10^5/30 mm well in DMEM:F12 supplemented with 5% CS. After 24 hr, the cells were washed 3 times with PRF-DMEM, and the medium was replaced with either PRF-SFM, PRF-SFM containing 20 ng/ml IGF-I or PRF-SFM with 5 ng/ml EGF. At days 0 (media change) and 2, the number of cells was determined by direct cell counting with the Trypan blue exclusion test.

Anchorage-independent growth assay

This assay was performed as previously described (Sell *et al.*, 1993). Briefly, the cells were plated at a concentration of 5×10^3/30 mm plate in DMEM with 10% FBS solidified with 0.2% agarose. DMEM with 10% FBS plus 0.4% agarose was used as an underlay. Colonies greater than 150 μm were scored after 3 weeks.

Apoptosis analysis

Flow cytometry cell sorting (FACS). At time 0, or after a 24 hr incubation in PRF-SFM media, cells were washed with cold PBS and fixed in 70% ice-cold ethanol. Following another wash in PBS, the cells were treated with RNase (75 μg/ml) for 30 min at 37°C, washed again in PBS and then resuspended in PBS containing 15 μg/ml propidium iodide. A minimum of 2×10^4 cells was analyzed by FACS with a Coulter Epics Profile II (Hialeah, FL).

In situ *detection of apoptosis.* Apoptotic cells were identified with the TACS/Blue Label *in situ* apoptotic detection kit (Trevigen, Gaithersburg, MD) following the manufacturer's protocol. Briefly, the cells were plated on glass slides in 100 mm plates and grown until 70% confluence. Then the cultures were washed 3 times with PRF-DMEM and shifted to PRF-SFM for 24 hr. Next, the cells were fixed in 3.7% paraformaldehyde and treated first with protease and then with H_2O_2 (to remove exogenous peroxidase). *In situ* labeling of fragmented DNA was performed with Klenow enzyme in the presence of labeled oligodeoxynucleotides. Labeled DNA was visualized with Blue Label, followed by counterstaining with Red Counterstain B. For each experimental condition, at least 1×10^3 cells were counted, and apoptosis was determined based on specific staining and cell morphology.

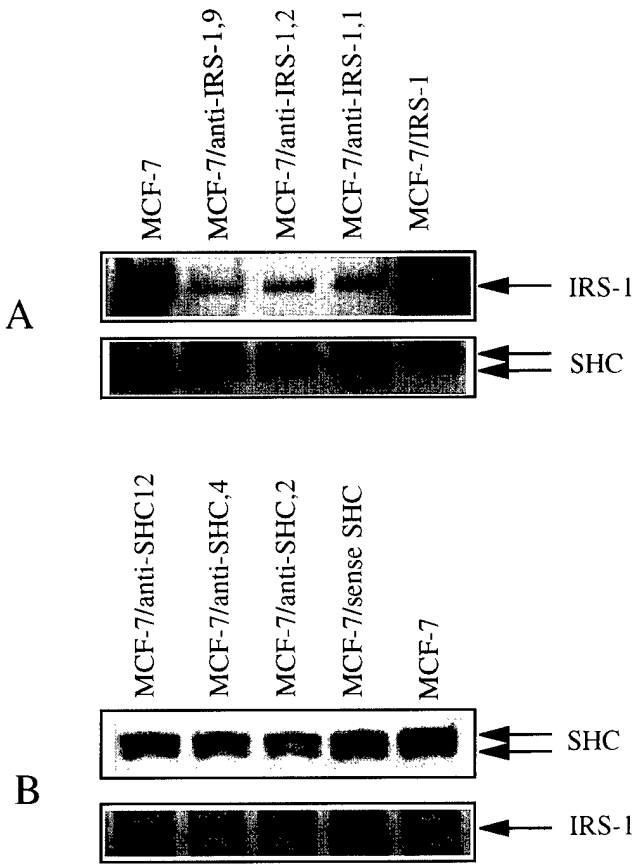

FIGURE 1 – Levels of IRS-1 and SHC in the developed clones. The levels of target proteins in MCF-7/anti-IRS-1 cells *(a)* and MCF-7/anti-SHC cells *(b)* and control cell lines were immunodetected as described in Material and Methods.

Cell aggregation assay

This assay has been performed as described before (Guvakova and Surmacz, 1997). Briefly, Matrigel (extracellular matrix) (Biocoat/Becton Dickinson, Bedford, MA) was reconstituted according to the manufacturer's instruction. Cells were plated at a concentration 2×10^4 cells/well in 24-well plates coated with 200 μl of Matrigel. After 6 days, the number and size of spheroids (aggregates) were counted and measured, and the cultures were photographed. To determine the number of viable cells, the spheroids were dissociated from the matrix during a 2 hr incubation in Dispase (Biocoat/Becton Dickinson) at 37°C, and the cells were counted with the Trypan blue exclusion test.

Cell motility assay

Cell motility was tested using Transwell polycarbonate membrane inserts with a 0.8 μm pore size (Corning/Cambridge, MA), as previously described (Doerr and Jones, 1996). The cells were plated in DMEM:F12 plus 5% CS at a concentration of 2×10^4 cells/insert. The inserts were placed in wells containing either DMEM:F12 plus 5% CS (controls), or DMEM:F12 plus 5% CS supplemented with either 20 ng/ml IGF-I or 5 ng/ml EGF. After a 16 hr incubation, the cells that traversed through the pores and attached to the underside of the insert were stained with Coomassie blue. The number of cells was determined by direct cell counting.

RESULTS

Development of MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones

To investigate the importance of IRS-1- and SHC-dependent signaling pathways in MCF-7 breast cancer cells, we developed, by stable transfection and selection in G418, several MCF-7-derived clones expressing antisense RNA to either IRS-1 or SHC. Ninety-five percent of the G418-resistant clones were PCR positive for plasmid integration; among these clones, approximately 25% exhibited an evident down-regulation of target protein. In MCF-7/anti-IRS-1 clones, the level of IRS-1 was reduced up to 85%, whereas in MCF-7/anti-SHC clones, up to 55% inhibition of SHC protein expression was observed. Interestingly, in both cases, we did not obtain clones with an intermediate (approx. 25–40%) degree of reduction. The levels of IRS-1 and SHC in several clones with the best inhibition of target protein expression are shown in Figure 1. In MCF-7/anti-IRS-1 clones 9, 2 and 1, the amounts of IRS-1 were reduced by 85%, 80% and 70%, respectively (Fig. 1*a*). In MCF-7/anti-SHC clones 12, 4 and 2, SHC expression (both p47 and p52) was inhibited by 47%, 50% and 55%, respectively (Fig. 1*b*). Notably, p66 SHC was undetectable in all MCF-7-derived cell lines, which confirmed our previous findings (Guvakova and Surmacz, 1997).

To control for specificity of antisense RNA activity, we measured the amounts of IRS-1 in MCF-7/anti-SHC clones and, conversely, the levels of SHC in MCF-7/anti-IRS-1 clones. The amounts of IRS-1 in all MCF-7/anti-SHC clones and MCF-7 cells were similar, with a variation of ±12%. Also, the levels of SHC were comparable in MCF-7/anti-IRS-1 clones and MCF-7 cells, with a variation of ±15% (Fig. 1*a,b,* lower panels).

The clones MCF-7/anti-IRS-1 2 and 9 and MCF-7/antisense SHC 2 and 4, exhibiting the best inhibition of target protein expression, were selected for further experiments.

MCF-7 cells with reduced levels of IRS-1 or SHC exhibit inhibition of monolayer growth

The ability of MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones to grow in monolayer culture was tested under 4 different conditions: DMEM:F12 plus 5% CS, PRF-SFM, PRF-SFM plus 20 ng/ml IGF-I, or PRF-SFM plus 5 ng/ml EGF (Fig. 2). The treatments with IGF-I and EGF were chosen because, of the many growth factors tested, these were the best mitogens for MCF-7 cells cultured in our laboratory (data not shown). Several control cell lines were used in this experiment: the parental MCF-7 cells, MCF-7/pc4 cells transfected with an empty vector (Guvakova and Surmacz, 1997) and MCF-7/IRS-1 cells characterized by overexpression of IRS-1 and amplification of IGF signaling (Surmacz and Burgaud, 1995). In a 2-day experiment, the growth of MCF-7 cells increased 50%, 70%, 54% and 80% in PRF-SFM, and PRF-SFM supplemented with IGF-I, EGF and CS, respectively. The increase in the number of MCF-7 cells under given condition was taken as 100%; the increase of the number of tested cells was calculated relative to MCF-7 cells. The growth was defined as increase in the number of viable cells. It should be noted that MCF-7 cells secrete IGF-like mitogens (Surmacz and Burgaud, 1995); therefore all experimental conditions included additional IGF-like autocrine factors.

In medium containing 5% CS (Fig. 2), the proliferation of MCF-7/anti-SHC clones was significantly inhibited. Specifically, relative to the parental cells, the growth was reduced by 55% (clone 2) and 27% (clone 4). Similarly, in MCF-7/anti-IRS-1 cells, the viable cell number was decreased by 61% (clone 2) and 57% (clone 9).

In PRF-SFM (Fig. 2), despite the presence of IGF-like autocrine factors, a large population of MCF-7/anti-IRS-1 cells was dying. In fact, compared with MCF-7 cells, the viable cell number was decreased by 142% (clone 2) and 130% (clone 9). Under the same conditions, MCF-7/anti-SHC clones survived better, although their growth was inhibited by 60% (clone 2) and 93% (clone 4).

Similar results were obtained in PRF-SFM supplemented with 20 ng/ml IGF (Fig. 2). Here, the growth of MCF-7/anti-SHC clones was inhibited by 67% (clone 2) and 83% (clone 4). Under these conditions, MCF-7/anti-IRS-1 cells were massively dying; relative to MCF-7 cells, a 145% (clone 2) and 148% (clone 9) decrease in cell number was noted.

In PRF-SFM supplemented with 5 ng/ml EGF (Fig. 2*d*), MCF-7/anti-SHC clones were inhibited by 82% (clone 2) and 74% (clone 4), while in MCF-7/anti-IRS-1 clones a 75% (clone 2) and 41% (clone 9) growth decrease was obtained.

The control cells, MCF-7/sense-SHC and MCF-7/pc4, grew like MCF-7 cells under all tested conditions. MCF-7/IRS-1 cells exhibited increased responsiveness to IGF-I and EGF, compared with the parental cell line, consistent with the previously published data (Surmacz and Burgaud, 1995).

All developed cell lines retained responsiveness to E2. In all tested cell lines, a 2-day stimulation with E2 alone caused an

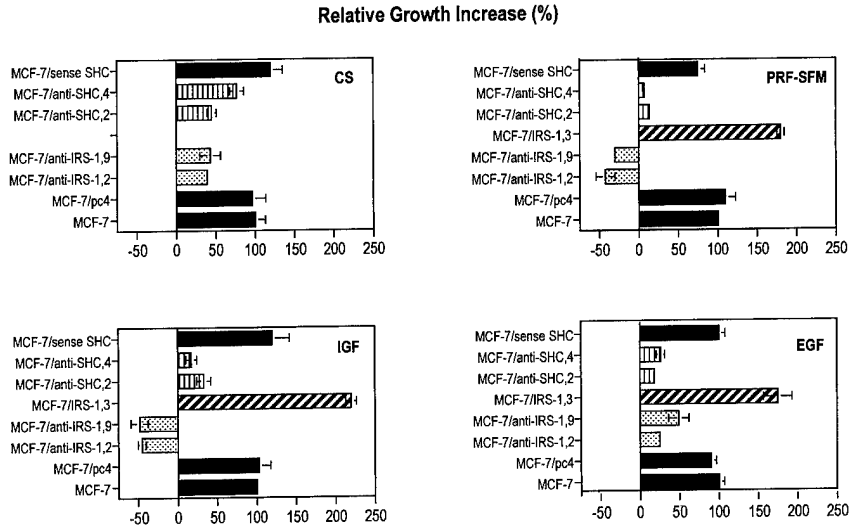

FIGURE 2 – Anchorage-dependent growth. Ordinate: relative percent increase in cell number, with the increase of MCF-7 cells taken as 100%. Abscissa: cell lines tested.

approximately 30% growth increase relative to the cell number at day 0 (data not shown).

MCF-7/anti-IRS-1 cells undergo apoptosis under serum-free conditions

To determine the mechanism of cell death apparent in monolayer growth in PRF-SFM and PRF-SFM plus IGF-I, the clones were analyzed for evidence of apoptosis. Two independent methods were employed, *in situ* detection of fragmented DNA and FACS analysis.

In growing cells (time 0), apoptosis was identified in a small fraction of all tested cell lines (Table I). In contrast, after 24 hr in culture of growth factor- and estrogen-reduced conditions, the rate of apoptosis considerably increased in MCF-7/anti-IRS-1 cells, up to 39.6%, but not in MCF-7/anti-SHC cells or other cell lines. Similar results were obtained after a 48 hr culture in PRF-SFM (data not shown). The higher incidence of apoptotic cell death in MCF-7/anti-IRS-1 clones was confirmed with FACS analysis, in which a pre-G_1 peak, possibly representing the subfraction of apoptotic cells, was observed (Fig. 3). In contrast, such a subfraction was undetectable in MCF-7 cells (Fig. 3) and MCF-7/anti-SHC clones (data not shown).

Anchorage-independent growth is blocked in MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells

The overexpression of IRS-1 has been shown to enhance anchorage-independent growth in MCF-7 cells (Surmacz and Burgaud, 1995). Amplification of SHC promoted transforming abilities in fibroblasts (Pelicci *et al.*, 1992). Here, we tested anchorage-independent growth (colony formation in soft agar) of MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones (Table II). In both cases, we found that colony formation was similarly inhibited, by at least 72%, when compared with MCF-7 cells. The anchorage-independent growth of control cell lines was comparable to that of MCF-7 cells.

MCF-7/anti-SHC clones exhibit impaired aggregation and survival on the extracellular matrix

Our previous results indicated that overexpression of the IGF-IR in MCF-7 cells markedly increased the ability of cells to aggregate on the extracellular matrix (Matrigel) (Guvakova and Surmacz, 1997). Moreover, the formation of multiple cell-cell contacts supported proliferation of clustered cells and decreased the rate of cell death. Here we studied whether reduced levels of IRS-1 or SHC (and, presumably, impaired signaling depending on these substrates) would affect cell-cell interaction on Matrigel.

The experiments demonstrated that while MCF-7/anti-IRS-1 clones were able to aggregate on ECM to a similar extent as control cell lines (MCF-7 and MCF-7/sense SHC cells), the aggregation of both MCF-7/anti-SHC clones was clearly inhibited (Fig. 4). Specifically, MCF-7, MCF-7/sense SHC cells and MCF-7/anti-IRS-1 clones produced large spheroids ranging in size from 230 to 300 μm, whereas MCF-7/anti-SHC clones formed small aggregates (approximately 50 μm in diameter). Furthermore, the clones that formed large spheroids (MCF-7, MCF-7/sense SHC and MCF-7/anti-IRS-1 cells) were also able to survive on ECM up to 7 days. In contrast, the population of viable MCF-7/anti-SHC cells was reduced by at least 50% during this period of time (Table III).

EGF-dependent cell motility is affected by the reduction of SHC levels in MCF-7 cells

The IGF-IR has been shown to mediate motility in breast cancer cells (Doerr and Jones, 1996). We studied the ability of MCF-7/anti-IRS-1 and MCF-7/anti-SHC cells to migrate toward a chemo-attractant, IGF or EGF (Table IV). Both growth factors stimulated the motility of all studied cell lines. The tendencies to migrate toward IGF were similar for all clones; however, some clonal variations were observed (64–95% increase over basal migration in growth medium). When EGF was used as an chemo-attractant, in MCF-7 cells, MCF-7/IRS-1 and MCF-7/sense-SHC clones, as well as in both MCF-7/anti-IRS-1 clones, migration increased by 28–56% over that stimulated by IGF (Table IV). In contrast, migration of MCF-7/anti-SHC clones toward EGF was decreased by 32% (clone 2) and 70% (clone 4) compared with IGF stimulation. In all cell lines tested, the differences between IGF-I and EGF chemo-attraction were statistically significant ($p < 0.05$, by ANOVA).

DISCUSSION

Although it is known that polypeptide growth factors, such as the IGFs and the ligands of the erbB family of receptors, play an important role in the regulation of breast cancer growth and progression, the functions of their different signaling pathways in the development of a neoplastic phenotype have not been elucidated. We have investigated the role of 2 signaling elements, IRS-1, a major substrate of the IGF-IR (but also involved in insulin and IL-4 signaling; Myers *et al.*, 1994) and SHC, an important substrate of different tyrosine kinase receptors, *e.g.*, the IGF-IR and erbB-type Rs (Sepp-Lorenzino *et al.*, 1996; Giorgetti *et al.*, 1994; Pelicci *et al.*, 1992). Since previous studies have demonstrated growth inhibition in MCF-7 cells stably expressing an IGF-IR antisense RNA (Neuenschwander *et al.*, 1995), we have used an antisense RNA approach to generate MCF-7 cell lines expressing reduced levels of either IRS-1 or SHC. The developed antisense clones were tested for their ability to grow under anchorage-dependent and -independent conditions, to survive in estrogen- and growth factor-reduced media, to migrate toward chemo-attractants and to develop and maintain cell-cell interactions on the extracellular matrix.

The major findings of this work are: 1) In MCF-7 cells, IRS-1 and SHC are involved in the regulation of monolayer and anchorage-independent growth; 2) significant reduction of IRS-1 levels is accompanied by cell death; 3) down-regulation of SHC levels affects cell-cell interactions on extracellular matrix; and 4) decrease of SHC levels impairs EGF-, but not IGF-I-stimulated migration of MCF-7 cells.

The most striking differences between MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones were seen in cell aggregation on Matrigel. The results suggested that normal amounts of SHC are required for the formation and maintenance of cell-cell contacts. We have demonstrated previously that in MCF-7 cells, E-cadherin-dependent cell-cell adhesion is significantly enhanced by the overexpression of the IGF-IR. Moreover, the IGF-IR and its substrates, IRS-1 and SHC, are able to associate with the E-cadherin complex (Guvakova and Surmacz, 1997). The mechanism of IGF-I-stimulated adhesion in breast epithelial cells remains unclear (Guvakova and Surmacz, 1997; Bracke *et al.*, 1993), but based on the present work, SHC signaling could be a contributing factor. The involvement of SHC in cell-cell interactions is also supported by the finding of a direct association of SHC and N-cadherin *in vitro* (Xu *et al.*, 1996).

TABLE I – APOPTOSIS IN MCF-7/ANTI/IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Apoptotic cells (%)[1]	
	0 hr	24 hr
MCF-7	0.8 ± 0.5	3.0 ± 0.8
MCF-7/pc4	0.5 ± 0.1	1.9 ± 0.1
MCF-7/IRS	1.2 ± 0.7	3.7 ± 1.1
MCF-7/anti-IRS-1, 2	1.9 ± 0.8	22.5 ± 2.3
MCF-7/anti-IRS-1, 9	4.3 ± 1.2	39.6 ± 1.4
MCF-7/anti-SHC, 2	1.2 ± 0.8	1.5 ± 0.3
MCF-7/anti-SHC, 4	0.8 ± 0.1	3.6 ± 0.5
MCF-7/sense-SHC	2.0 ± 0.2	2.3 ± 0.0

[1]DNA fragmentation *in situ* was detected using a Trevigen *in situ* detection kit following the manufacturer's methodology, as described in Material and Methods. The results shown are means ± SD from at least 3 independent experiments.

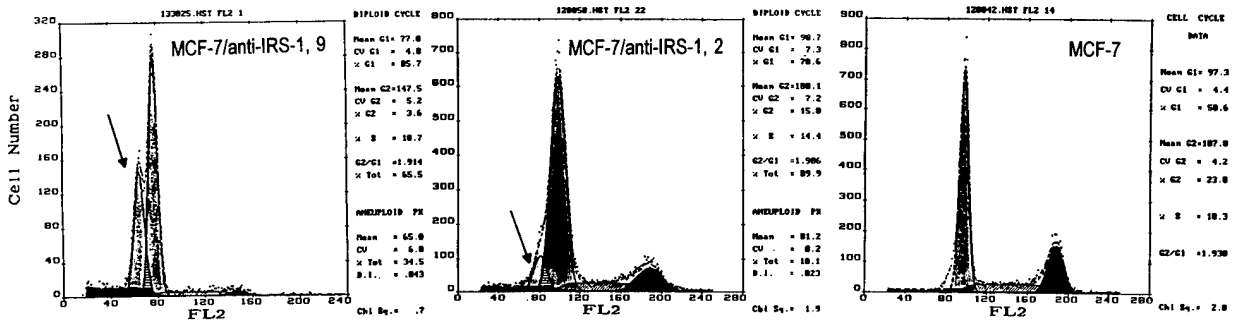

FIGURE 3 – Apoptosis analysis. To identify apoptosis in MCF-7 cells and MCF-7/anti-IRS-1 clones 2 and 9, FACS analysis was performed with a Coulter Epics Profile II as described in Material and Methods. The arrows indicate pre-G_1 peaks, representing apoptotic cell fractions in MCF-7/anti-IRS-1, 9 and MCF-7/anti-IRS-1, 2 clones. Subsequent to pre-G_1 (left to right), are the peaks representing G_1, S, and G_2 cell subpopulations.

TABLE II – ANCHORAGE-INDEPENDENT GROWTH OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Number of colonies[1]	Inhibition (%)
MCF-7	101 ± 5.9	—
MCF-7/pc4	98 ± 1.0	3
MCF-7/anti-IRS1, 2	14 ± 6.7	86
MCF-7/anti-IRS-1, 9	28 ± 4.5	72
MCF-7/anti-SHC, 2	12 ± 3.5	88
MCF-7/anti-SHC, 4	28 ± 6.0	72
MCF-7/sense SHC	103 ± 9.4	—

[1]Cells were plated in soft agar in 10% FBS at 5×10^5 cells/plate. Colonies greater than 150 μm were counted after 3 weeks. The data are means ± SD from 3 independent experiments.

Remarkably, in MCF-7 cells, down-regulation of IRS-1 levels, which was correlated with the inhibition of the growth in monolayer culture and in soft agar, did not affect cell aggregation, and only moderately (20%) inhibited cell survival on Matrigel. A limited role of IRS-1 in cell-cell adhesion is also suggested by the fact that overexpression of this molecule does not improve aggregation in MCF-7/IRS-1 cells; however, it does prolong cell survival on Matrigel (data not shown). The latter suggest a role of IRS-1 in protection from cell death. This function of IRS-1 has also been demonstrated in the present work; in particular, MCF-7/anti-IRS-1 cells cultured as monolayer in PRF-SFM and PRF-SFM with IGF-I, were massively dying. This suggested that other pathways activated under these conditions, for instance SHC, did not provide sufficient signal for survival and could not compensate for IRS-1 loss. Importantly, in anti-IRS-1 clones, cell death was executed through apoptosis. Apoptosis was detected by FACS and *in situ* labeling, the methods of choice for breast epithelial cells in which a classical apoptotic DNA ladder is usually undetectable (Wilson *et al.,* 1995). Apoptosis was not identified in cells with normal IRS-1 amounts, for instance, in MCF-7 cells or in anti-SHC clones growing in the presence of IGF (autocrine or exogenous). Interestingly, when anti-IRS-1 clones were cultured in media supplemented with CS or EGF, the cells were able to survive, possibly due to the activation of some IRS-1-independent anti-apoptotic mechanisms. For example, a PI-3 kinase pathway (which can be activated directly by the EGFR) has been found to control cell survival (Parrizas *et al.,* 1997).

The role of SHC in survival of MCF-7 cells is difficult to evaluate, partly because in our model, reduction of SHC levels was not as great as that of IRS-1. The fact that the inhibition of SHC by approximately 60% was not sufficient to induce cell death in monolayer culture, even in PRF-SFM, indicates that normal amounts of SHC were not essential for survival under these conditions. However, the survival of cells on Matrigel (in the presence of different growth factors) was inhibited in MCF-7/anti-SHC clones. We speculate that this phenomenon represented a secondary effect to the impaired cell aggregation in these cells, since aggregation itself has been shown to promote survival on Matrigel (Guvakova and Surmacz, 1997).

The studies on anchorage-dependent growth also suggested an important function of both SHC and IRS-1 in cell proliferation. In MCF-7/anti-IRS-1 and MCF-7/anti-SHC clones, cell growth was blocked even in medium containing CS. This reflected mostly the inhibition of proliferation, since, even in MCF-7/anti-IRS-1 cells, only minimal cell death was observed (Table I and data not shown). Similar results were obtained in medium supplemented with EGF (naturally containing autocrine IGF-like factors and possibly other unidentified mitogens) (Fig. 2). The greatest extent of growth reduction, for both MCF-7/anti-IRS-1 and MCF-7/anti-SHC, was seen in SFM containing only IGF (autocrine or exogenous). The results suggested that normal levels of either IRS-1 or SHC were not sufficient to sustain growth in IGF when the other pathway was (presumably) impaired. Therefore, both substrates must control IGF-I-dependent monolayer growth of MCF-7 cells.

Both SHC and IRS-1 also appear to be critical in the maintenance of anchorage-independent growth since colony formation in both MCF-7/anti-SHC and MCF-7/anti-IRS-1 clones was significantly (by at least 70%) inhibited, compared with control cell lines expressing normal amounts of both substrates (Table II).

It has been shown that in different breast cancer cell lines, motility is stimulated by the activation of the IGF-IR (Doerr and Jones, 1996). Our results did confirm that IGF-I stimulates migration of MCF-7 cells. We also found that in MCF-7 cells, migration was stimulated by EGF. Contrary to Doerr and Jones (1996), in our hands EGF was a significantly stronger chemoattractant for the cells studied than IGF. The reason for this discrepancy is unclear. It is possible that the subline of MCF-7 cells cultured in our laboratory differs from the one described by others; in particular, our MCF-7 cells were able to traverse only uncoated membranes, whereas the cells described by Doerr and Jones (1996) invaded through either gelatin, laminin or collagen.

Under our experimental conditions, IGF-I-dependent migration was similar in all tested cell lines and was not significantly inhibited in either MCF-7/anti-SHC or MCF-7/anti-IRS-1 clones. It is possible that the IGF-IR activated other pathways providing sufficient signal for migration, or, alternatively, the extent of the inhibition of either IRS-1 or SHC signaling was insufficient to inhibit migration. It is noteworthy that the EGF-stimulated motility was significantly blocked in MCF-7/anti-SHC clones, suggesting that SHC may act as a critical signaling substrate of the EGFR-regulated migration.

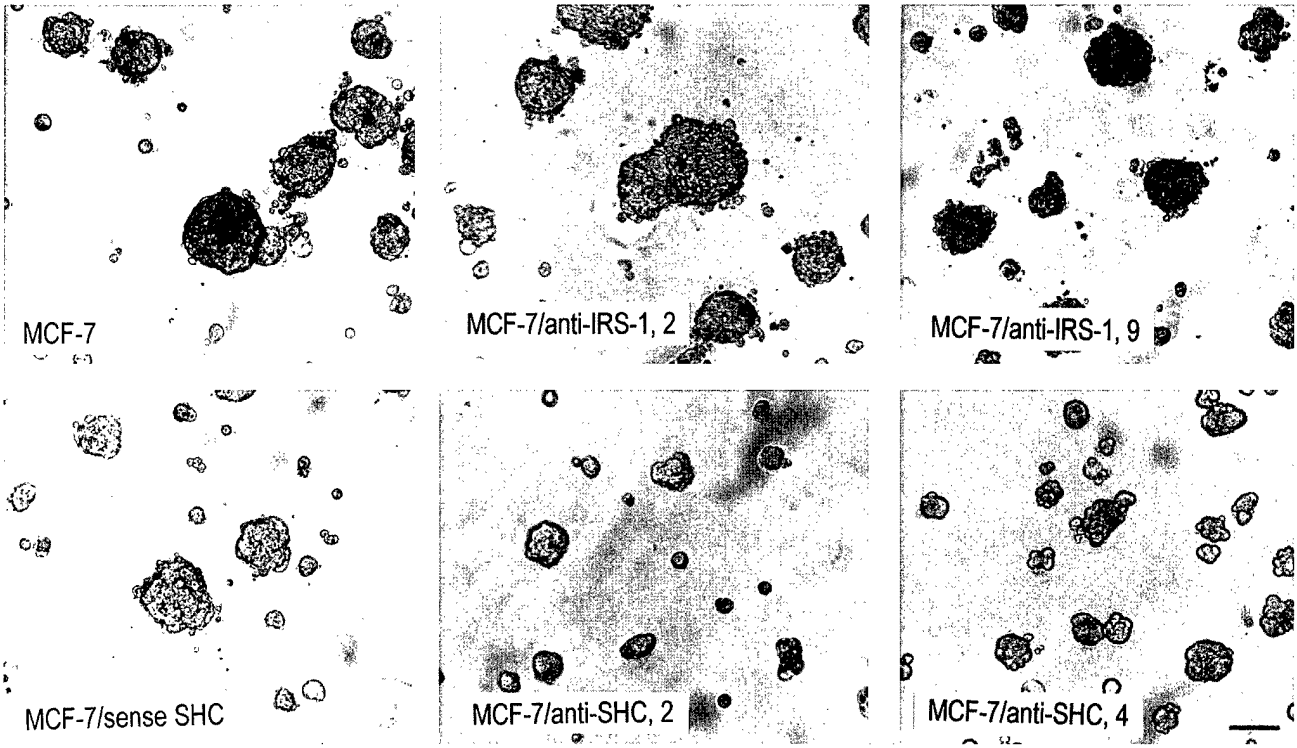

FIGURE 4 – Cell aggregation on Matrigel. Representative photographs of cell aggregation in cell lines tested. The aggregation was tested as described in Material and Methods; cells were photographed with 100× magnification on day 5 of the experiment. Scale bar = 100 μm.

TABLE III – SURVIVAL OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CLONES ON MATRIGEL

Cell lines	Number of cells at day 6[1]
MCF-7	19,300 ± 818
MCF-7/pc4	17,266 ± 1,010
MCF-7/anti-IRS-1, 2	18,233 ± 709
MCF-7/anti-IRS-1, 9	18,366 ± 1,517
MCF-7/anti-SHC, 2	6,000 ± 1,000
MCF-7/anti-SHC, 4	9,500 ± 1,040
MCF-7/sense SHC	19,333 ± 1,527

[1]Cells were plated at 2×10^4/well in 24-well plates on Matrigel Matrix (Biocoat/Fisher). On day 6, the number of cells was determined by direct cell counting with Trypan blue exclusion after dissociation of aggregates by Dispase at 37°C for 2 hr. The data are means ± SD from at least 3 independent experiments.

TABLE IV – MOTILITY OF MCF-7/ANTI-IRS-1 AND MCF-7/ANTI-SHC CELLS

Cell line	Chemo-attraction (% over basal)[1]	
	IGF	EGF
MCF-7	195 ± 7.2	245 ± 7.1
MCF-7/IRS-1	186 ± 6.1	217 ± 2.5
MCF-7/sense SHC	167 ± 6.1	212 ± 10.0
MCF-7/anti-IRS-1, 2	164 ± 3.0	194 ± 2.6
MCF-7/anti-IRS-1, 9	165 ± 5.0	195 ± 9.0
MCF-7/anti-SHC, 2	175 ± 2.0	148 ± 5.9
MCF-7/anti-SHC, 4	195 ± 8.6	128 ± 7.0

[1]Cells (2×10^4) suspended in growth medium were plated in Transwell inserts, and the migration toward IGF or EGF was evaluated as described in Material and Methods. Migration toward growth medium was taken as basal. The data are average ± SD from at least 3 independent experiments.

In summary, our results point to the importance of 2 post-receptor signaling molecules, IRS-1 and SHC, in the maintenance of the neoplastic phenotype in breast epithelial cells; the results also suggested that these substrates may have distinct functions in breast cancer cell biology.

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Overexpressed IGF-I Receptors Reduce Estrogen Growth Requirements, Enhance Survival, and Promote E-Cadherin-Mediated Cell–Cell Adhesion in Human Breast Cancer Cells

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The insulin-like growth factor I receptor (IGF-IR) paracrine or autocrine loop plays an important role in the maintenance of breast cancer growth. Cancer cells contain several-fold higher levels of the IGF-IR than normal breast tissue; however, it is still not clear whether abnormally high activation of IGF-IR signaling may induce progression of the disease. To address this question, we have established several MCF-7-derived clones (MCF-7/IGF-IR cells) overexpressing the IGF-IR. We report here that overexpression of the IGF-IR did not modify sensitivity of cells to IGF-I; however, responsiveness to the ligand was moderately enhanced in most of the MCF-7/IGF-IR clones (measured by [^{3}H]-thymidine incorporation into DNA). All MCF-7/IGF-IR clones responded to the synergistic action of 1 n*M* estradiol (E2) and small amounts of IGF-I (up to 0.8 ng/ml). Exposure of cells to higher concentrations of IGF-I abolished estrogen requirements for stimulation of DNA synthesis in all MCF-7/IGF-IR clones, but not in the parental cells. The most important finding of this work was that the amplification of the IGF-IR induced cell–cell adhesion in MCF-7 cells. High levels of the IGF-IR promoted cell aggregation on Matrigel, allowed proliferation of cells within the aggregates, and protected clustered cells from death. In both MCF-7 and MCF-7/IGF-IR cells, IGF-I stimulated aggregation, whereas an anti-E cadherin antibody blocked cell–cell adhesion. Furthermore, immunofluorescence staining with specific antibodies revealed co-localization of the IGF-IR and E-cadherin at the points of cell–cell contacts. Moreover, the IGF-IR and its two substrates, insulin receptor substrate 1 and SHC, were contained within the E-cadherin complexes. Our results suggest that overexpressed IGF-IRs, by promoting the aggregation, growth, and survival of breast cancer cells, may accelerate the increase of tumor mass and may also prevent cell scattering.

INTRODUCTION

The insulin-like growth factor I receptor (IGF-IR)[2] belongs to the tyrosine kinase receptor superfamily [1]. Upon ligand binding, the intrinsic tyrosine kinase of the IGF-IR is activated, which results in the immediate tyrosine phosphorylation of several cellular substrates. Two well-characterized substrates are insulin receptor substrate-1 (IRS-1) and SHC [2–5]. Both act as docking proteins, recruiting different effector molecules and activating multiple signaling systems, for instance, the pathways of Ras or PI-3 kinase. Ultimately, some of the IGF-IR-induced signals stimulate nuclear events, while others are involved in the reorganization of cell morphology [2–9].

Several lines of evidence support an important role of the IGF system (the IGF-IR, its ligands, and IGF-binding proteins) in breast cancer. IGF-I and IGF-II are potent mitogens for cultured breast cancer cells [10] and the levels of the IGF-IR are significantly higher in breast tumors than in normal breast tissue [11]. In primary breast cancer, a correlation has been found between tumor size, the levels of IRS-1, and recurrence of the disease [12]. In estrogen-responsive breast cancer cells, physiological concentrations of estradiol (E2) upregulate the expression of IGF-IRs [13, 14], IGF-II [15], and certain binding proteins [16]. On the other hand, overexpression of IGF-II or IRS-1 renders cells estrogen-independent [14, 17]. Most importantly, blockade of the IGF-IR autocrine or paracrine loop with anti-IGF-IR antibodies, excess of IGF-binding protein, antisense RNA against IGF-IR, or antisense oligonucleotides against IRS-1 inhibits breast cancer growth *in vitro* or *in vivo* [14, 18–20].

The role of the IGF-IR in the metastasis of mammary tumor is not clear. In mouse mammary carcinoma, cells

[1] To whom correspondence and reprints requests should be addressed at Kimmel Cancer Institute BLSB 606A, Thomas Jefferson University, 233S 10th Street, Philadelphia, PA 19107. Fax: (215) 923-0249.

[2] Abbreviations used: E2, 17-β-estradiol; FACS, fluorescence-assisted cell sorting; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IRS-1, insulin receptor substrate 1; MCF-7/IGF-IR, MCF-7 cells overexpressing IGF-IRs; NS, statistically nonsignificant; PCR, polymerase chain reaction; SFM, serum-free medium; PRF-SFM, phenol red-free SFM.

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with higher levels of IGF-IRs are more metastatic [21]. On the other hand, based on the studies on IGF-IR content in breast tumors, it has been postulated that higher levels of the receptor are associated with a more favorable clinical outcome and may reflect a more differentiated breast cancer phenotype [11].

It is well established that in breast cancer cells, the acquisition of a metastatic phenotype may be related to deterioration or deactivation of adherens-type cell junctions. These types of junctions are structured around transmembrane cadherin proteins [22–26]. The strength of cadherin-mediated adhesion is regulated by different cytoplasmic catenins which connect cadherins to the actin filament network [24]. E-cadherin is often expressed in breast epithelial cells [22–24] and its presence usually correlates with a nonmetastatic phenotype [23–25]. On the other hand, loss or downregulation of E-cadherin has been observed in several metastatic breast cancer cell lines [23–25]. In several studies, restoration of E-cadherin function, by overexpression of this molecule or by treatment with growth factors or other compounds, resulted in increased adhesion and reduced metastasis [26–29]. Thus, an invasion suppresser role for E-cadherin has been postulated [28].

Here we report that in MCF-7 breast cancer cells, overexpressed IGF-IRs reduce estrogen requirements for growth and enhance responsiveness to low concentrations of IGF-I in the presence of E2. We also demonstrate that high levels of the IGF-IR promote cell–cell adhesion, allow proliferation of cells within aggregates, and protect clustered cells from death.

MATERIALS AND METHODS

IGF-IR expression plasmid. The pcDNA3/IGF-IR expression vector contains a full human IGF-IR cDNA [1] cloned into *Xho–Xba* polylinker sites of the pcDNA3 expression plasmid (Invitrogen). The expression of IGF-IR cDNA is driven by the CMV promoter. The plasmid also encodes a neomycin resistance gene.

Development of cell lines and cell culture conditions. MCF-7 cells were routinely grown in DMEM:F12 (1:1) containing 5% CS. In experiments that required estrogen-free conditions, cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM FeSO4, and 2 mM L-glutamine (PRF-SFM).

The MCF-7/IGF-IR cell lines used in this study were developed by stable transfection with plasmids pcDNA3/IGF-IR or pcDNA3, followed by selection in medium containing 2 mg/ml G418. The neomycin-resistant clones were then screened by PCR. MCF-7/IGF-IR clones were maintained in culture for no longer than 3 months in the above medium with addition of 200 μg/ml G418.

PCR. The incorporation of the IGF-IR cDNA into MCF-7 cells' genome was assessed by PCR. Briefly, DNA was isolated from 1000 to 10,000 cells. A fragment of the IGF-IR DNA was amplified using the following primers: upstream primer (located in the exon I of the IGF-IR coding sequence) 5′-AAG GAA TGA AGT CTG GCT CC-3′; downstream primer 5′-CTC GAT CAC CGT GCA GTT CT-3′ (in exon II). Using these primers we were able to discriminate between the endogenous and the transfected IGF-IR DNAs. The conditions of PCR were 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min; the last extension was for 6 min. The size of the DNA fragment amplified from the transfected IGF-IR cDNA was 170 bp.

FACS. To estimate the level of the IGF-IR in each of the MCF-7/IGF-IR clones, we used fluorescence-activated flow cytometry sorting (FACS) [30]. Briefly, the cells were cultured for 3 days in PRF-SFM (to downregulate endogenous receptors). Then the cells were trypsinized, washed with ice-cold PBS, and incubated for 30 min at 4°C in PBS containing 10 μg/ml of an anti-IGF-IR antibody (alpha-IR3, Oncogene Science). Next, the cells were washed with ice-cold PBS and incubated in the dark for 30 min at 4°C with 2 μg/ml of an FITC-conjugated goat anti-mouse IgG (Oncogene Science). Unbound antibody was removed by washing with PBS and the level of fluorescence was determined with EPICS Profile Analyzer. The primary antibody was omitted in control experiments.

Scatchard analysis. The number of the IGF-IR and ligand/receptor dissociation constant was determined by ligand replacement assay [30]. Briefly, the cells were plated at 1×10^5 cells per well in a 12-well plate in DMEM:F12 containing 5% CS. Next day, the cells were shifted to PRF-SFM for 3 days. Then, the cells were washed with DMEM and incubated for 6 h at 4°C in binding buffer [30] containing 0.1 nM ^{125}I-IGF-I (NEN/DuPont) and increasing concentrations of unlabeled IGF-I (at a range 0.0–5.0 nM). Next, the cells were washed three times with PBS containing 1 mg/ml BSA and lysed with 0.03% SDS. Cell-bound radioactivity was measured using a gamma counter. Nonspecific binding was determined in the presence of 33.0 nM unlabeled IGF-I. The binding characteristic was analyzed by the LIGAND program. For all tested cell lines, the best fit of binding was obtained with a one-site model.

Binding competition assay. The IGF-I binding assay was performed in the presence of insulin [31]. The cells were plated and synchronized in SFM as described for Scatchard analysis and then incubated with 0.1 nM ^{125}I-IGF-I and unlabeled insulin at concentrations from 0.01 to 100 nM. The same amounts of unlabeled IGF-I were used in control experiments. The amount of cell-bound ^{125}I-IGF-I was determined as described above.

Western blotting and immunoprecipitation. The levels of IGF-IR, IRS-1, SHC, and E-cadherin, as well as the levels of tyrosine phosphorylation of these proteins, were measured by Western blotting. The cells were cultured as described in the figure legends and then lysed, as described in Refs. [30, 32]. The protein lysate (300–1000 μg) was immunoprecipitated with an appropriate antibody. The following antibodies were used for immunoprecipitation: for IGF-IR, an anti-IGF-IR monoclonal antibody alpha-IR3 (Oncogene Science); for IRS-1, an anti-IRS-1 polyclonal antibody (UBI); for SHC, an anti-SHC polyclonal antibody (Transduction Laboratories); and for E-cadherin, an anti-E-cadherin monoclonal antibody (Transduction Laboratories). The immunoprecipitates were resolved by PAGE and the proteins were immunodetected with the appropriate antibody. For IRS-1, SHC, and E-cadherin, the antibodies were the same as used for immunoprecipitation. To detect the IGF-IR, we used an anti-IGF-IR polyclonal antibody (Santa Cruz). Tyrosine phosphorylation of the above proteins was detected by immunoblotting with an anti-phosphotyrosine monoclonal antibody PY20 (Transduction Laboratories). The amounts of proteins were estimated by laser densitometry reading. Tyrosine phosphorylation level of E-cadherin-associated proteins was measured 72 h after stimulation (which was the time necessary to complete and stabilize cell–cell aggregation).

[^{3}H]Thymidine incorporation. Cells were plated into 96-well plates at a concentration of 1×10^4 cells per well in DMEM:F12 containing 5% CS and were grown until 80% confluent. Then, the medium was replaced with PRF-SFM containing 10 nM tamoxifen (to synchronize the cells in quiescence). After 3 days, the cells were washed twice with PRF-SFM and incubated in PRF-SFM supplemented with different amounts of IGF-I, E2, or IGF-I + E2 for 18–20 h. Next, the cultures were pulsed with 0.5 μCi/well of [^{3}H]-thymidine for 4 h. The amount of radioactivity incorporated into

trichloroacetic acid-insoluble material was counted using a beta counter (1209 Rackbeta, Wallac). The stimulation of [^{3}H]thymidine incorporation into DNA was calculated as follows: cpm's obtained in unstimulated cells (PRF-SFM) were taken as basal value equaling 100%; cpm's in cells stimulated with either IGF-I, E2, or IGF-I + E2 were expressed as the percentage increase over basal level.

The sensitivity index ED_{50} was calculated as described in Ref. [33].

Soft agar assay. The anchorage-independent growth was determined by soft agar assay as described previously [14]. The growth of cells was tested in semisolid DMEM:F12 medium supplemented with either 10 or 2% FBS, as well as in PRF-SFM supplemented with 200 ng/ml IGF-I. Colonies of a size greater than 150 μm were counted after 2 and 3 weeks.

Aggregation on Matrigel. Matrigel (Biocoat/Fisher) was reconstituted according to the manufacturer's instructions. The matrix (200 μl/well) was placed in a 24-well plate and allowed to solidify. A cell suspension of 2×10^4 cells in DMEM:F12 plus 5% CS was plated in each well and cultured for up to 21 days. The cultures were photographed at Days 5 and 11. At Day 16, the cells were released from the matrix using Dispase (Biocoat/Fisher), stained with trypan blue, and counted in a hemocytometer.

In several experiments, the cells were cultured on Matrigel in the presence of 50 ng/ml IGF-I to assess the ligand-dependent increase of aggregation. The requirement for E-cadherin was tested with the anti-E-cadherin antibody HECD-1 (Zymed) which was added at the time of plating at a concentration of 10 μg/ml.

Invasion assay. The invasiveness of MCF-7/IGF-IR cells was studied using 24-well invasion chambers (Biocoat/Fisher). Cells (2×10^4) suspended in DMEM:F12 with 5% CS were placed in the upper chamber and cultured for 24 or 48 h. Lower chambers contained the same growth medium. The number of cells that invaded the extracellular matrix and migrated to the underside of the chamber was determined by direct counting (after staining with 0.5% crystal violet). In several experiments, the medium in the lower chamber was supplemented with IGF-I (50–200 ng/ml) or E2 (10 nM) used as chemoattractants.

Immunofluorescence microscopy. The double staining for the IGF-IR and E-cadherin was performed on monolayer cultures of MCF-7 and MCF-7/IGF-IR cells. The localization of the IGF-IR was detected using indirect immunofluorescence, as recommended by Transduction Laboratories (protocol 9). Briefly, 70% confluent cells grown on glass coverslips were fixed for 10 min at room temperature (RT) in 3.7% formaldehyde, washed with PBS, treated with 0.2% Triton X-100 for 5 min, and then blocked in 0.2% BSA for 5 min. The fixed cells were incubated for 1 h at RT with 10 μg/ml of a rabbit polyclonal anti-human IGF-IR antibody (Santa Cruz), washed with PBS, and incubated with an anti-rabbit-lissamine/rhodamine-conjugated goat IgG (26 μg/ml) for 30 min. The localization of E-cadherin determined on the same slides using a monoclonal antibody HECD-1 (10 μg/ml) and a goat anti-mouse IgG-FITC-conjugated (2 μg/ml). The primary antibodies were omitted in control experiments.

Statistical analysis. The statistical evaluation of results was done using ANOVA single-factor analysis of variance. The significance level was taken as $P \leq 0.05$.

RESULTS

Development of MCF-7/IGF-IR clones. The MCF-7/IGF-IR clones were developed by stable transfection of MCF-7 cells with the pcDNA3/IGF-IR expression vector. The transfected cells were selected in 2 mg/ml G418. Forty-four G418-resistant clones were analyzed by PCR to detect the cells with the integrated IGF-IR plasmid and by FACS to identify the clones that overexpressed the IGF-IR. Ultimately, five MCF-7/IGF-IR clones were obtained, designated C-12, C-34, C-21, C-17, and C-15. In parallel, by stable transfection of MCF-7 cells with the pcDNA3 vector, we generated control clones MCF-7/pc 2 and MCF-7/pc 4.

The number of IGF-I receptors in MCF-7/IGF-IR clones was determined by Scatchard analysis (Fig. 1B). The cells were incubated for 4 days in phenol red-free serum-free medium (PRF-SFM) to ensure downregulation of endogenous IGF-IRs in the absence of E2. The IGF-IR content in MCF-7/IGF-IR clones ranged from 0.5×10^6 to 3.0×10^6 receptors/cell, which represented an 8- to 50-fold increase over the IGF-IR level in MCF-7 cells (0.6×10^5 receptors/cell) [14] (Figs. 1A and B). The number of receptors in the control MCF-7/pc 2 and pc 4 clones was slightly lower than that in the parental cells (0.3×10^5 and 0.4×10^5 sites/cell, respectively) but the differences did not reach statistical significance. The dissociation constant (K_d) in all clones was in the range of K_d values reported for IGF-I/IGF-IR binding [34–36] (Fig. 1A). In addition, in order to rule out the possibility that the increased number of binding sites was due to selective formation of IGF-I/insulin hybrid receptors [31], binding competition assays with insulin were performed. In the all MCF-7/IGF-IR clones, IGF-I binding was not displaced even with very high (100 nM) concentrations of insulin (data not shown).

Figure 1C demonstrates the IGF-IR levels in MCF-7/IGF-IR clones by FACS. Compared with MCF-7 cells, the increase in relative IGF-IR fluorescence in MCF-7/IGF-IR clones was from 2.5- to 25.1-fold. MCF-7/pc 2 and pc 4 clones exhibited fluorescence similar to that in MCF-7 cells (0.9 and 0.95 of the level in the parental cells).

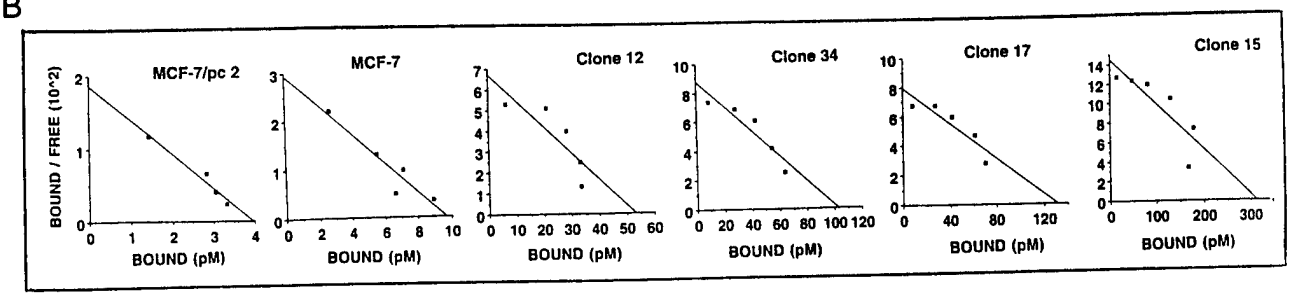
Additionally, the level of the IGF-IR protein in the developed clones was assessed by immunoprecipitation and subsequent immunoblotting with an anti-IGF-IR antibody (Fig. 2A). The increase of the IGF-IR beta-subunit in the clones C-12, C-34, C-21, C-17, and C-15 compared with that in MCF-7 cells was 2-, 5-, 7-, 10-, and 21-fold, respectively (estimated by laser densitometry). The levels of IGF-IR protein in clones MCF-7/pc 2 and pc 4 were similar to those in MCF-7 cells and were barely detectable by Western blotting (not shown).

In MCF-7/IGF-IR clones, both basal and IGF-I-induced tyrosine phosphorylation of the IGF-IR and IRS-1 were markedly increased compared with the levels in the parental cells. The representative experiment, involving clones with the highest overexpression of the receptor, is shown in Fig. 2B. In MCF-7/IGF-IR clones 15 and 17, the levels of tyrosine phosphorylation of the IGF-IR were consistently at least 4-fold higher in cells treated for 5 min with IGF-I, and at least 8-fold higher under SFM, than the corresponding levels in MCF-7

A

Cells	MCF-7/pc 2	MCF-7	Clone 12	Clone 34	Clone 21	Clone 17	Clone 15
N_R	0.03×10^6	0.06×10^6	0.5×10^6	0.9×10^6	0.8×10^6	1.1×10^6	3.0×10^6
K_d	0.21	0.28	0.79	1.16	0.47	1.65	2.1
K_{FACS}	0.9	1.0	2.5	2.8	6.9	12.6	25.1

B



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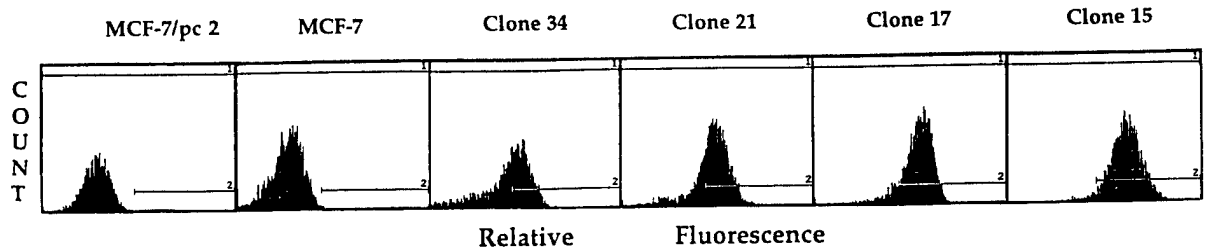


FIG. 1. Characteristics of MCF-7/IGF-IR clones. (A) Summary of parameters: IGF-IR number (N_R), dissociation constant K_d (nM), and relative fluorescence index (K_{FACS}). Binding parameters were determined, and FACS analysis was performed, as described under Materials and Methods. K_{FACS} represents the fold increase of the relative IGF-IR fluorescence in MCF-7/IGF-IR clones over that in MCF-7 cells. (B) Scatchard analysis. Abscissa, bound IGF (pM); ordinate, bound/free IGF $\times 10^{-2}$. The binding experiments for each clone were repeated at least three times. The representative Scatchard plots are shown. (C) FACS analysis. Abscissa, relative IGF-IR fluorescence; ordinate, cell number. In each experiment, 5×10^3 of cells were analyzed. Each analysis was performed at least two times. The representative results are shown. In control experiments, where cells were stained with secondary antibody only, the level of fluorescence was undetectable.

cells (estimated by laser densitometry). The tyrosine phosphorylation of IRS-1 was also more pronounced in MCF-7/IGF-IR clones than in the parental cells: specifically, at least 2-fold greater under IGF treatment, and from 1.6- (clone 12) to 6-fold (clone 15) greater in SFM (Fig. 2C). In the latter case, presumably due to a low concentration of ligand, there was an apparent correlation between tyrosine phosphorylation of IRS-1 and the number of IGF-IRs. In the presence of IGF-I, the saturation of IRS-1 stimulation was obtained in clone 12 expressing 5×10^6 receptors/cell. The levels of IRS-1 protein in the studied cells were similar under all experimental conditions (not shown).

E2 responsiveness in MCF-7/IGF-IR cells. MCF-7 cells and all developed MCF-7/IGF-IR clones responded similarly to E2 with stimulation of [^{3}H]thymidine incorporation into DNA (data not shown). In all cell lines, 0.05 nM E2 yielded a maximal response (a 2.9- to 6.8-fold increase over the basal level), while concentrations up to 1.0 nM had no additional effect. The differences in E2 responsiveness observed among the tested clones were not statistically significant. E2 at a concentration higher than 20.0 nM exerted an inhibitory effect. Consequently, a concentration of 0.1 nM E2 was used in all further experiments.

Sensitivity and responsiveness of MCF-7/IGF-IR cells to IGF-I alone or in combination with E2. The overexpression of IGF-IR did not modify sensitivity to IGF-I (evaluated by [^{3}H]thymidine incorporation into DNA) (Fig. 3). Specifically, the ED_{50} for MCF-7 was 0.56 ng/ml and for MCF-7/IGF-IR cells it ranged from 0.42 to 0.67 ng/ml, P = NS. The responsiveness to IGF-I, however, was increased in most MCF-7/IGF-IR clones. For instance, with 4 ng/ml IGF-I, there was a 325% increase of [^{3}H]thymidine incorporation in MCF-7 cells, whereas increases of 336, 492, 580, 728, and 648% were observed in clones C21, C12, C34, C17, and C15, respectively.

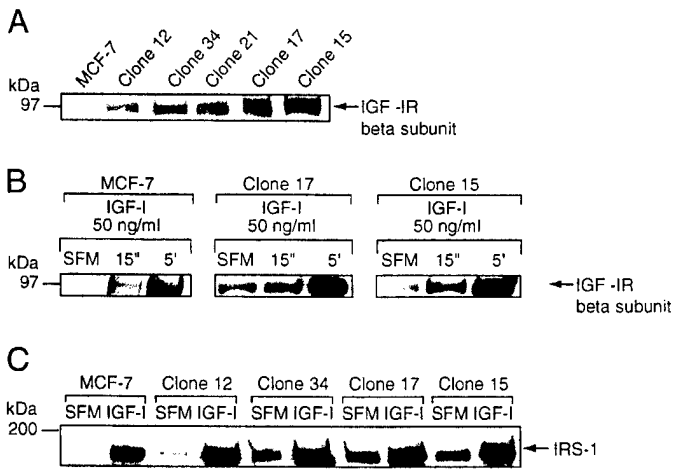


FIG. 2. Activation of IGF-IR signaling in MCF-7/IGF-IR cells. (A) IGF-IR protein levels. The IGF-IR protein level in MCF-7 cells and clones 12, 34, 21, 17, and 15 was determined by immunoprecipitation with an anti-IGF-IR antibody and subsequent Western immunoblotting, as described under Materials and Methods. 300 μg and 1 mg of proteins were used to immunoprecipitate the IGF-IR from MCF-7/IGF-IR and MCF-7 cells, respectively. The analysis was performed at least two times for each clone. (B) Tyrosine phosphorylation of the IGF-IR. Cells were incubated in PRF-SFM for 3 days and then stimulated with 50 ng/ml IGF-I for 15 s or 5 min. Tyrosine phosphorylation of the IGF-IR was measured by immunoprecipitation with an anti-IGF-IR antibody followed by Western immunoblotting with an anti-phosphotyrosine antibody. The experiments were repeated at least four times; representative results for MCF-7 cells, clone 17, and clone 15 are shown. (C) Tyrosine phosphorylation of IRS-1. Cells were grown in PRF-SFM for 3 days with or without 50 ng/ml of IGF-I. 300 μg of lysate was immunoprecipitated with an anti-IRS-1 antibody and immunoblotted with an anti-phosphotyrosine antibody. The experiments were repeated at least two times; representative results are presented.

In MCF-7 cells, 0.1 n*M* E2 combined with IGF-I (0.01–100 ng/ml) produced a synergistic effect on [^{3}H]-thymidine incorporation. In contrast, in all MCF-7/IGF-IR clones, E2 increased the mitogenic response only in the presence of low concentrations of IGF-I (less than 1 ng/ml). With larger amounts of IGF-I, the synergistic effect of E2 was abolished, and IGF-I alone was sufficient to maximally stimulate DNA synthesis (Fig. 3). The effect of IGF-I alone or in combination with E2 on DNA synthesis in MCF-7/pc clones was similar to that found for MCF-7 cells (not shown).

Overexpression of the IGF-IR does not improve the ability of MCF-7 cells to grow in soft agar or in monolayer culture. The ability of MCF-7/IGF-IR clones to grow under anchorage-independent conditions was similar to that exhibited by MCF-7 and MCF-7/pc cells. Specifically, all tested cell lines formed approximately 100 colonies in soft agar containing 10% FBS, 120 colonies in agar with 10% FBS plus 200 ng/ml IGF-I, and 45 colonies in agar with 2% FBS. In addition, similarly to the parental cell line, none of the MCF-7/IGF-IR cells produced colonies greater than 100 μm in semisolid PRF-SFM supplemented with 200 ng/ml IGF-I.

In monolayer culture, the increase in cell number was similar in all studied cell lines, regardless of the level of IGF-IR overexpression. Specifically, 72 h after stimulation with IGF-I (0.1–10 ng/ml), in all cases, the number of cells increased ~1.7 ± 0.3-fold (data not shown).

Overexpression of the IGF-IR does not induce invasiveness of MCF-7 cells in vitro. MCF-7 cells have been reported as noninvasive or poorly invasive [27]. We found that the overexpression of IGF-IR in MCF-7 cells did not alter their noninvasive phenotype *in vitro*. Using invasion chambers (Biocoat/Fisher), we noted that only approximately 0.2% cells were able to traverse the extracellular matrix. Additionally, invasiveness of the cells was not stimulated when IGF-I or the combination of IGF-I + E2 was used as chemoattractant.

Overexpression of the IGF-IR stimulates cell–cell adhesion in MCF-7 cells. MCF-7 cells cultured on Matrigel (extracellular matrix) formed globular aggregates characteristic of a noninvasive phenotype [27]. As shown in Fig. 4, cell aggregation was significantly stimulated in clones overexpressing the IGF-IR. The extent of aggregation appeared to parallel the increase of the IGF-IR number in the cells (Figs. 4a–4e). For instance, at Day 5 of culture, MCF-7 cells did not produce any aggregates of a size greater than 150 μm, whereas the clones C-12 and C-34 (Figs. 4b and 4c) formed an average of 8 and 10 such aggregates, respectively. The clones 17 and 15 (Figs. 4d and 4e) produced a few clusters of a size greater than 300 μm and several smaller aggregates of the size 150–300 μm. The average results from five experiments were as follows: for clone C-17, there were 4 aggregates greater than 300 μm and 5 of the size 150–300 μm, and for clone 15, there were aggregates greater than 300 μm and 7 of the size 150–300 μm.

When culture on Matrigel was extended up to 21 days, MCF-7 cells and the clones expressing less than 1.1×10^6 IGF-IRs progressively disaggregated and died (Fig. 4f–4h). At Day 16 of the experiment, of 2×10^4 initially plated cells, we found an average of 1.2×10^2, 1×10^2, and 8×10^3 viable cells for MCF-7, clone 34, and clone 12, respectively. In contrast, the clones expressing the highest levels of the IGF-IR not only remained well aggregated, but also proliferated in compact clusters (Figs. 4i–4j). Indeed, at Day 16, the number of cells was even increased up to 1.1×10^5 cells for clone 17 and to 4.6×10^4 cells for clone 15.

The aggregation and survival of MCF-7/pc 2 and pc 4 cells cultured on Matrigel was similar to that noted for the parental cells. The morphology and size of the clusters produced by MCF-7/pc 2 cells is shown in Fig. 5A.

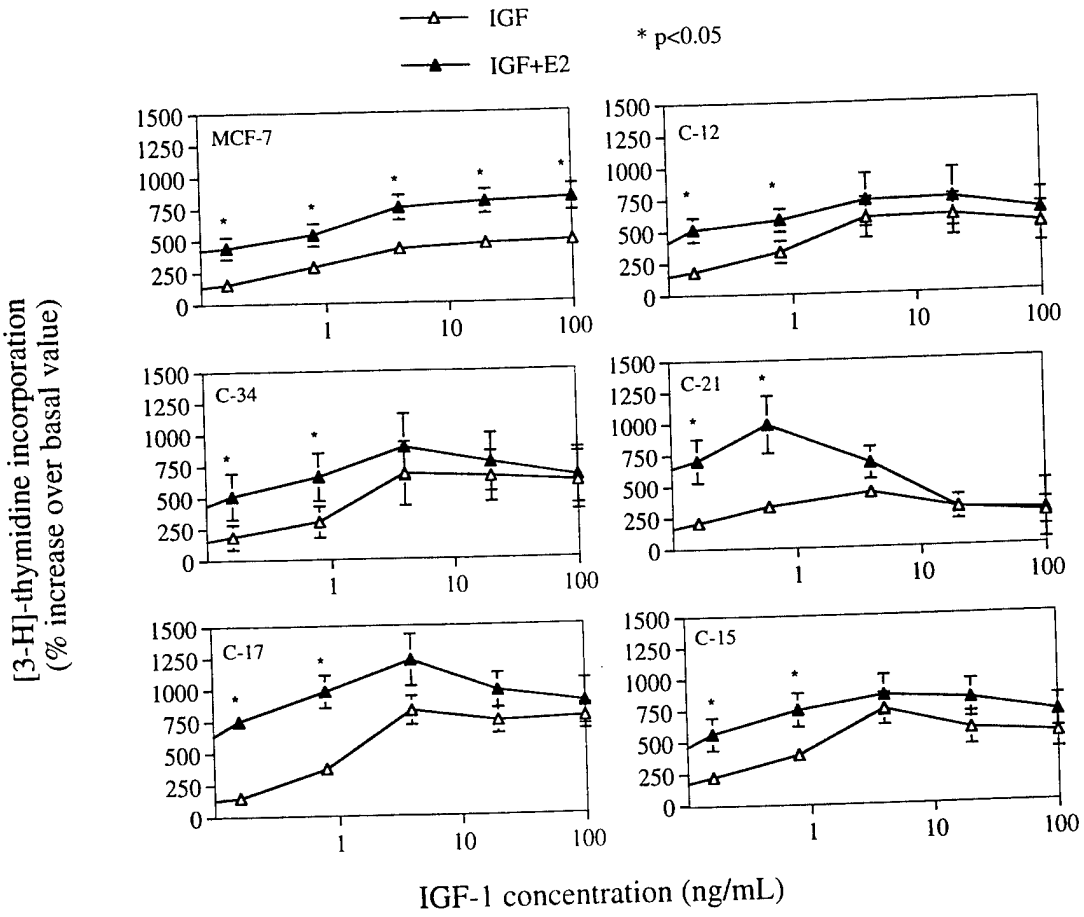


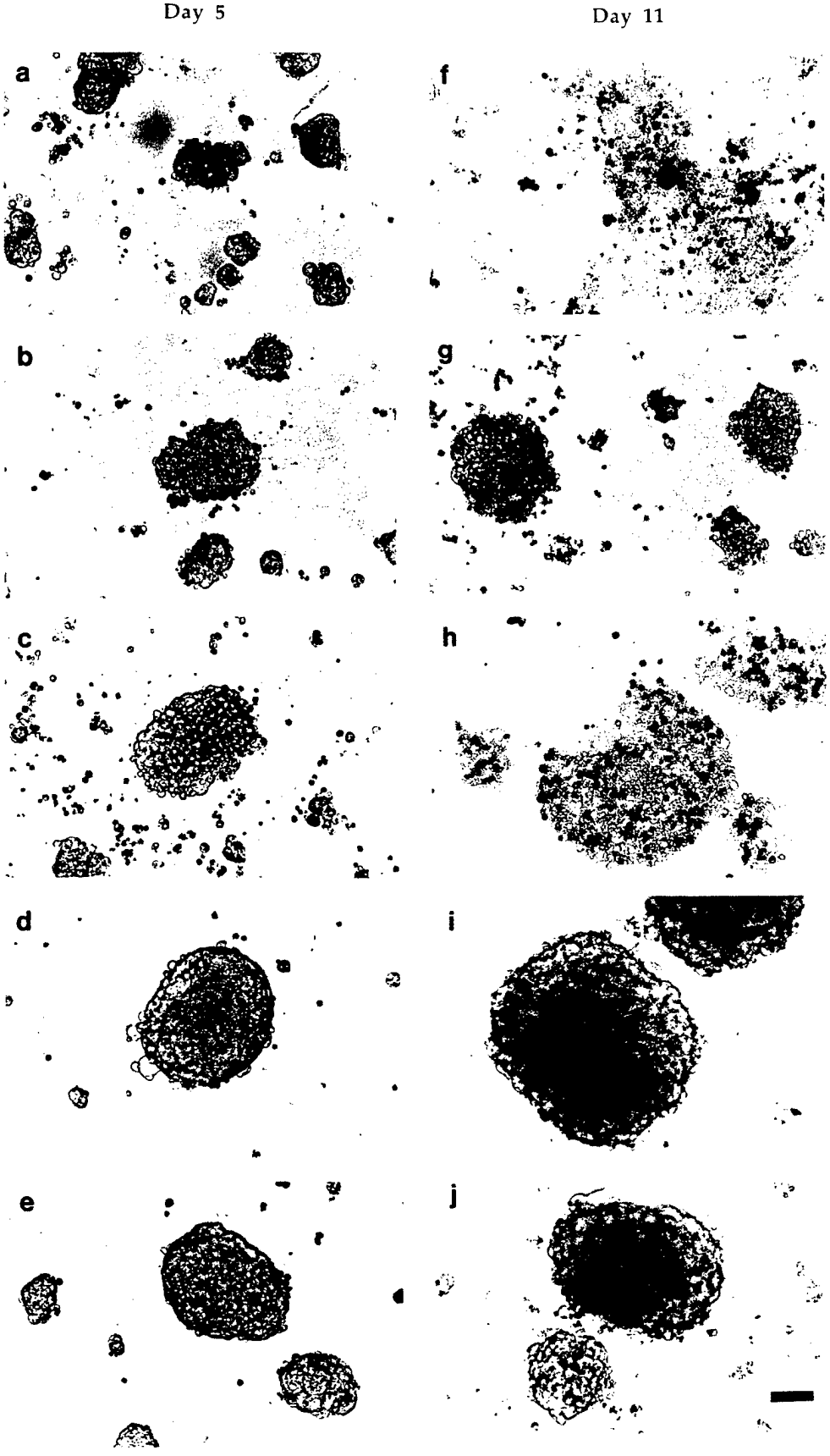
FIG. 3. Stimulation of [^{3}H]thymidine incorporation by IGF-I alone or in combination with E2 in MCF-7 and MCF-7/IGF-IR cells. T cells were synchronized and stimulated with different amounts of IGF-I with or without 0.1 n*M* E2, as described under Materials a Methods. Abscissa, tested concentrations of IGF-I. Ordinate, percentage increase of [^{3}H]thymidine incorporation (in cpm) over basal le (cpm values in untreated cells). The values are means from at least four experiments. Bars, SE. Asterisks indicate statistically signific differences between IGF-I and IGF-I plus E2 values by ANOVA ($P < 0.05$).

IGF-I stimulates aggregation of MCF-7 and MCF-7/IGF-IR cells: Anti-E-cadherin antibody blocks the formation of aggregates. In the presence of 20 ng/ml IGF-I, MCF-7 cells as well as MCF-7/IGF-IR clones displayed increased aggregation on Matrigel. As shown in Fig. 5, the size of average aggregates typically increased from approximately 100 to 150 μm in MCF-7 cells (Fig. 5B, parts a and c), and from 180 to 250 μm in clone 15 (Fig. 5B, parts b and d). In contrast, in all studied cell lines, the addition of an anti-E-cadherin antibody at the time of cell plating effectively blocked cell–cell adhesion. The effect of anti-E-cadherin antibody on aggregation in MCF-7 cells and in MCF-7/IGF-IR clone 15 is presented in Fig. 5B, parts e and f.

The IGF-IR co-localizes with E-cadherin in MCF-7/IGF-IR cells. The IGF-IR and E-cadherin were d tected by double-staining with specific antibodies. MCF-7 cells, immunofluorescence analysis with anti-IGF-IR antibody produced barely visible staini on the cell surface (Fig. 6a), while immunodetecti with anti-E-cadherin antibody revealed the typical cadherin honeycomb pattern [25] (Fig. 6b).

In MCF-7/IGF-IR clones, the IGF-IR was easily tectable by immunofluorescence. The staining peared to be concentrated at areas of cell–cell conta (Figs. 6c and 6e), which was particularly evident clones with very high receptor content (Fig. 6e). I markably, in MCF-7/IGF-IR clones, the IGF-IR co

FIG. 4. Aggregation of MCF-7/IGF-IR cells. 20,000 cells were plated on Matrigel. The morphology of the clones was recorded by ph contrast microscopy after 5 and 11 days of culture. MCF-7 cells (a, f); clone 12 (b, g); clone 34 (c, h), clone 17 (d, i); clone 15 (e, j). Bar μm. The experiments were repeated at least four times for all analyzed cells. The representative results are shown.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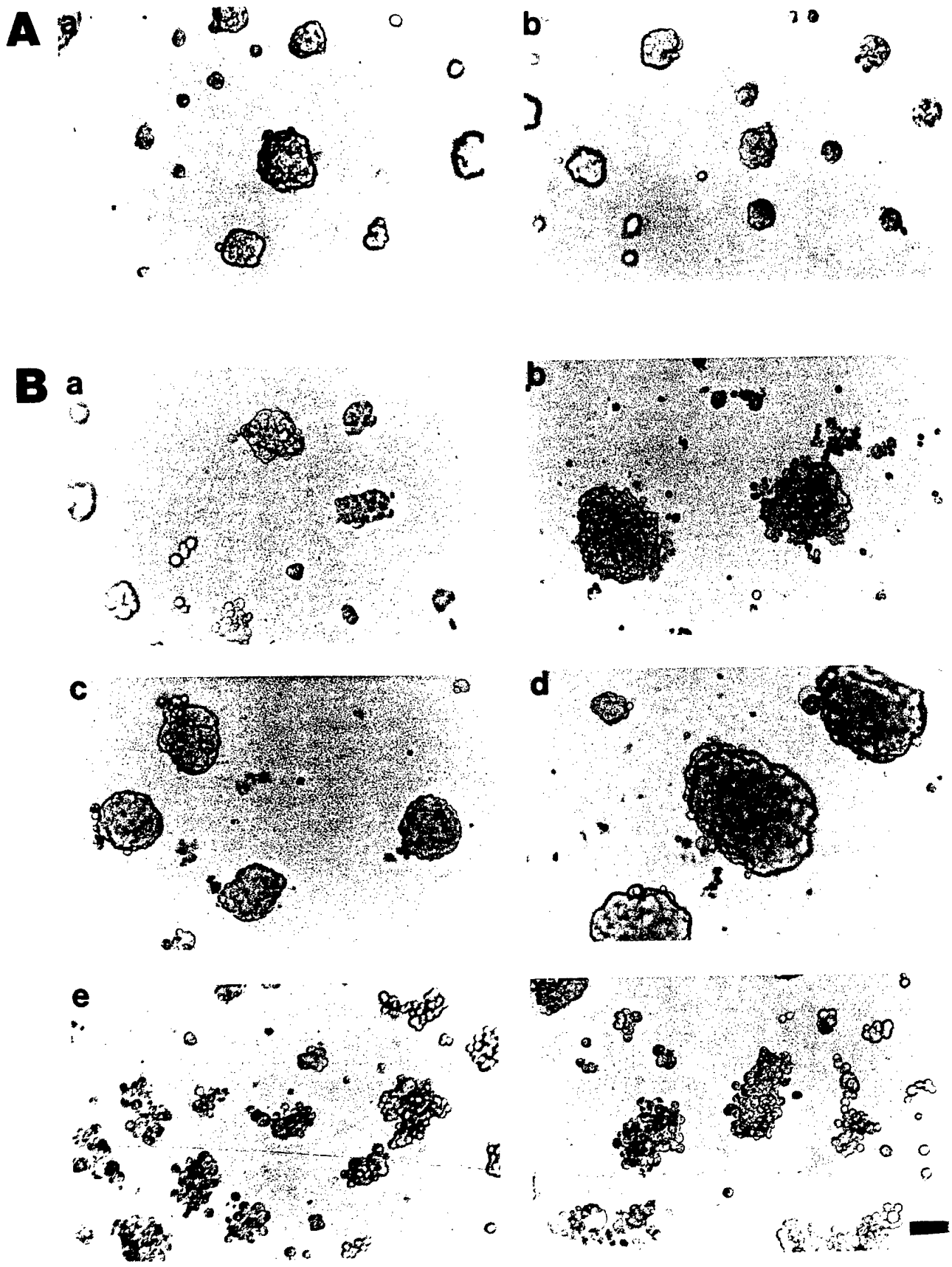
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calized with E-cadherin (Figs. 6d and 6f). Interestingly, in contrast to the uniform membrane distribution of E-cadherin in MCF-7 cells, the localization of this protein appeared to be altered in cells with very high IGF-IR content. Specifically, more E-cadherin tended to accumulate in the areas of cell membranes containing the highest amounts of the IGF-IR (Figs. 6e and 6f).

IGF-IR, IRS-1, and SHC associate with E-cadherin. We further investigated whether the IGF-IR and associated substrates physically interact with E-cadherin complexes. Figure 7 shows representative data from several independent experiments. Figure 7A demonstrates patterns of tyrosine phosphorylation of proteins coprecipitating with E-cadherin in MCF-7 cells and in clones 12, 17, and 15 cultured under various conditions. In all analyzed cells, the patterns of tyrosine phosphorylated proteins associated with E-cadherin were similar (Fig. 7A) and contained at least seven distinct bands. The most prominent were the bands of the approximate sizes 95, 185, and 200 kDa (the last not seen in MCF-7 cells). In the following experiments, we examined, by reprobing the blots with different antibodies, whether E-cadherin-associated proteins contain the elements of the IGF-I signaling pathway. Due to multiple stripping and reprobing, we were not always able to demonstrate the levels of all studied proteins in each individual blot. However, in several independent experiments, we confirmed the presence of each of the IGF-IR signaling molecules, the IGF-IR, IRS-1, and SHC, in E-cadherin complexes of all studied cells (not shown).

Figure 7B demonstrates the amounts of E-cadherin, IRS-1, IGF-IR, or SHC in the precipitates. E-cadherin, as expected, was detectable in all precipitates. In MCF-7 cells, a 185-kDa band noticeable in cells treated with IGF-I (Fig. 7A) contained IRS-1. IRS-1 was also present in E-cadherin precipitates of other cells (shown here for clone 15). In contrast to MCF-7 cells, however, tyrosine phosphorylation of IRS-1 in MCF-7/IGF-IR clones 12, 17, and 15 was also noticed under SFM or 5% CS conditions.

The E-cadherin complexes contained the IGF-IR (shown here for MCF-7 cells, clone 12, and clone 17). Relative to E-cadherin levels, the amount of IGF-IRs in the complexes appeared to be greater in cells overexpressing the receptor (see clones 12 and 17, compared with MCF-7 cells). The extent of tyrosine phosphorylation of the IGF-IR was impossible to determine since the phosphorylated band of the size 95 kDa contained not only the IGF-IR but also large amounts of β-catenin which was phosphorylated on tyrosine residues (not shown).

The tyrosine phosphorylation of E-cadherin was low and was further reduced (approximately 50%) in MCF-7, clone 12, and clone 15, as a result of 72 h of IGF-I treatment (Fig. 7). In several experiments, we did not notice any consistent modification of E-cadherin protein expression under IGF-I. The exception was clone 17, in which IGF-I caused a reduction of E-cadherin levels.

SHC proteins complexed with E-cadherin were not phosphorylated on tyrosines under any of the experimental conditions.

DISCUSSION

Although numerous studies [10–20] have suggested an important role for the IGF paracrine or autocrine loop in the regulation of breast cancer growth, the impact of the IGF-IR on the progression of the disease is still largely unknown. Here, we examined whether a substantial increase in the IGF-IR levels would induce processes associated with breast cancer progression, such as increased sensitivity or responsiveness to IGF-I, development of estrogen independence, enhancement of anchorage-independent growth, or induction of invasiveness. In IGF-I- and E2-dependent, noninvasive MCF-7 cells, the overexpression of the IGF-IR produced: (i) a moderate mitogenic effect, reflected by sensitization of cells to low concentrations of IGF-I in the presence of E2 and reduction of E2 requirements with large amounts of IGF-I, and (ii) a marked morphogenic effect, reflected by stimulation of cell–cell adhesion.

Our studies were greatly facilitated by the successful development of MCF-7-derived clones expressing different levels of IGF-IRs. The first part of this work provides characteristics of our experimental model. The expression of IGF-IRs was determined by three independent methods: Scatchard binding assay (Fig. 1B), FACS analysis (Fig. 1C), and Western immunoblotting (Fig. 2). The number of IGF-I binding sites in the developed clones assessed by Scatchard assay was from 8- to 50-fold higher than that in the parental cells (Figs. 1A and 1C). This higher IGF-I binding was not associated with any noticeable overexpression of low-affinity binding sites for IGF-I (likely to represent surface IGF binding proteins [37] (Fig. 1B). Hybrid IGF-I/insulin receptors, with affinity toward both li-

FIG. 5. (A) Aggregation of a control clone. The morphology of control cells MCF-7 (a) and MCF-7/pc2 (b) at Day 5 of culture on Matrigel. Three independent experiments were performed. (B) Effects of IGF-I and anti-E-cadherin antibody on aggregation of MCF-7 and MCF-7/IGF-IR cells. The sizes and morphology of average aggregates produced by MCF-7 cells (a–e) and MCF-7/IGF-IR clone 15 (b–f) cultured in control medium were analyzed on Day 5 of the experiment. In parallel, the cells were grown in the presence of 50 ng/ml IGF-I (MCF-7, c; clone 15, d) or 10 μg/ml of anti-E-cadherin antibody (HECD 1) (MCF-7, e; clone 15, f).

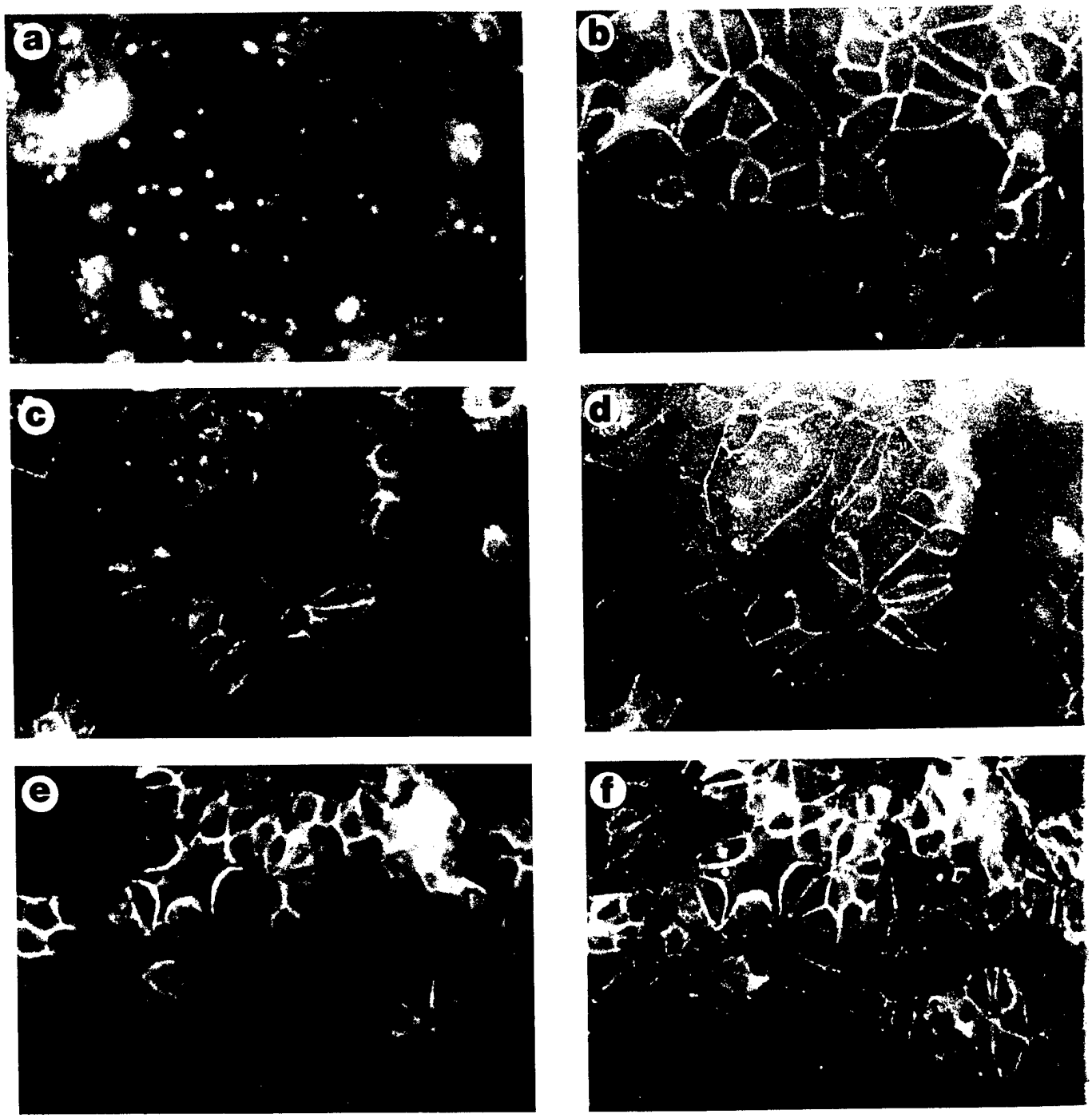

FIG. 6. Co-localization of the IGF-IR and E-cadherin in MCF-7/IGF-IR cells. The IGF-IR was detected with an anti-IGF-IR polyclo antibody and anti-rabbit rhodamine-conjugated IgG. E-cadherin was localized with an anti-E-cadherin monoclonal antibody (HECD-1) a anti-mouse FITC-conjugated IgG, as described under Materials and Methods. The localization of the IGF-IR in MCF-7 cells (a), clone (c), clone 15 (e) and the staining for E-cadherin in MCF-7 (b), clone 12 (d) and clone 15 (f) were examined and photographed under a Ze axiophot microscope with an original magnification of ×400. The arrowheads indicate specific staining. The fluorescence staining observ in the cell nucleoli in a, c, and e was nonspecific.

gands, have been described in many models [31]. We established, by competition binding, that in the MCF-7/IGF-IR clones, IGF-I, but not insulin, was the principal ligand for the overexpressed receptors.

The overexpression of the IGF-IR in MCF-7/IGF-I cells was further confirmed by two semiquantitati methods, FACS analysis and Western immunoblottin By FACS, the amount of the IGF-IR protein in t

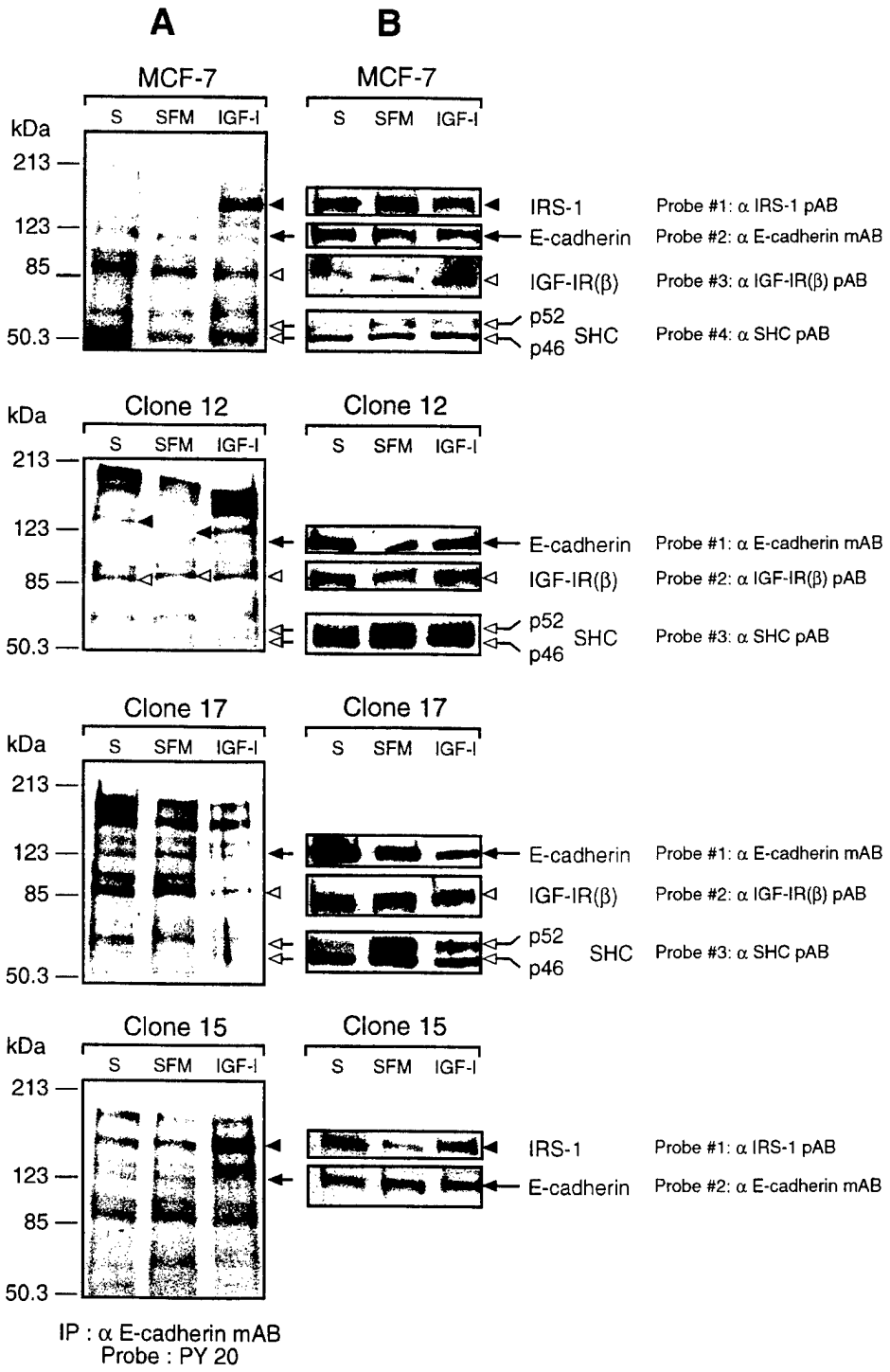

FIG. 7. Association of IGF-I signaling molecules with E-cadherin. Cells were incubated for 3 days in DMEM:F12 with 5% CS (S), PRF-SFM (SFM) or PRF-SFM with 50 ng/ml IGF-I (IGF-I) and then lysed. (A) Tyrosine phosphorylation of E-cadherin-associated proteins. 500 μg of protein lysate was immunoprecipitated with 4 μg of an anti-E-cadherin monoclonal antibody. (B) Protein levels of E-cadherin and associated proteins. The immunoblots shown in (A) were stripped and reprobed with an anti-E-cadherin antibody. Next, the membranes were stripped again and reprobed with either an anti-IRS-1 or an anti-IGF-IR antibody. Subsequently, the blots probed with an anti-IGF-IR antibody were stripped and hybridized with an anti-SHC antibody. Due to multiple stripping and the resulting decrease in membrane-bound proteins, we were not able to demonstrate the levels of all studied proteins in a single blot. Note: the E-cadherin and IGF-IR bands in the clone 12 blot were shifted because of a gel crack.

developed clones was elevated from 2.5- to 25.1-fold compared with MCF-7 cells (Fig. 1C); Western blotting revealed a 2- to 21-fold increase (Fig. 2A). Furthermore, we provided evidence that in MCF-7/IGF-IR cells, the extent of IGF-I-induced tyrosine phosphorylation of both IGF-IR and IRS-1 was higher than in MCF-7 cells. Interestingly, a saturation of IGF-stimulated tyrosine phosphorylation was observed. In the case of the receptor, the maximum was seen in cells expressing approximately 1×10^6 sites/cell, whereas expression of 5×10^5 sites/cell produced maximal tyrosine phosphorylation of IRS-1. While the latter may reflect the limited abundance of IRS-1 in cells, the limiting step for the former remains unknown.

All developed clones retained similar sensitivity to E2, which strongly suggested that transfection of MCF-7 cells with the IGF-IR did not affect E2 receptor expression. Similar observations have been reported by other investigators who studied MCF-7 cells overexpressing the EGFR, IGF-IR, or c-erbB-2 [36, 38, 39].

In the next step, we tested monolayer growth of MCF-7/IGF-IR clones with special emphasis on their responsiveness to mitogenic effects of IGF-I and E2. We also examined cell growth under anchorage-independent conditions. Unexpectedly, in all MCF-7/IGF-IR clones, even those with the highest overexpression, the sensitivity to IGF-I was not significantly changed, and the responsiveness to this ligand was only moderately increased, compared with the corresponding parameters in MCF-7 cells. The cells with higher levels of IGF-IRs, however, exhibited increased responsiveness to low concentrations of IGF-I in the presence of E2. With higher concentrations of IGF-I, the synergistic effect of IGF-I and E2 was abolished, despite the fact that the cells retained normal E2 sensitivity. Consequently, in most MCF-7/IGF-IR clones, IGF-I alone, at a concentration of 4 ng/ml, produced the maximal mitogenic effect independent of E2. Our results are in agreement with the [illegible]t work of Daws *et al.* [36], who studied MCF-[illegible]xpressing approximately 2 $\times 10^5$ IGF-IRs [illegible]ls, although the combined mitogenic eff[illegible]d E2 was not increased, an enhanced [illegible]ow concentrations of IGF-I in the pre[illegible] was noted. Interestingly, in MCF-[illegible]despite moderately increased respon-[illegible]NA synthesis to IGF-I, the growth rate [illegible] cell number) was not enhanced compared [illegible]-7 cells.

[illegible] above observations suggest that in breast epi-[illegible] cells, the mitogenic effect mediated through the [illegible]-IR may be a saturated process. The exact nature of this phenomenon is not known. We speculate that in MCF-7/IGF-IR cells, the limited increase of response to IGF-I may be related to the translocation of a significant fraction of IGF-IRs to cell–cell boundaries where they are not fully accessible for IGF-I stim tion (Fig. 6) (see below).

Under anchorage-independent conditions, pa loss of E2 requirements has been described in br cancer cell lines overexpressing IGF-II [17] or II [14]. In contrast, breast cancer cells overexpres IGF-IRs generated by Daws *et al.* [36] as well as by laboratory retained E2 requirement for growth in agar. It is possible that in both cases, the exter receptor overexpression, or the abundance of avail receptors, was not sufficient to override E2 depende It cannot be excluded, however, that in breast ca cells, anchorage-independent growth requires act tion of specific E2 signaling, which can be overric by amplified IGF-II or IRS-1 signaling, but not IGI signaling.

In the following part of this work, we assessed i sive properties of MCF-7/IGF-IR cells *in vitro* as as the effects of IGF-IR overexpression on cell–cel hesion. MCF-7 cells are poorly invasive [25] but t invasive properties *in vitro* can be enhanced, for stance, by the overexpression of H-ras [37–40]. demonstrated that overexpression of the IGF-IR not stimulate invasiveness of MCF-7 cells. In contr we observed that high levels of IGF-IRs actually moted cell–cell adhesion and formation of large ganoid-like structures on Matrigel (Fig. 4). Im tantly, in this assay, the extent of aggregation (nun and sizes of aggregates) paralleled the level of IGF- Moreover, the cells expressing more than 1×10^6 s cell not only survived in the aggregates for the lon period but also continued to multiply within the for clusters. This suggested that the IGF-IR, by stimu ing cell–cell adhesion, promoted proliferation of ag gated cells and protected cells from death.

The role of the IGF-IR in cell–cell adhesion was ther substantiated by the evidence that IGF-I sti lated the aggregation of MCF-7 and MCF-7/IGI cells (Fig. 5B). The aggregation of these cells blocked in the presence of anti-E-cadherin antib which demonstrated that IGF-IR-enhanced adhe requires functional E-cadherin (Fig. 5B). Our find are consistent with the data of Bracke *et al.* [26], demonstrated that in the invasive breast cancer line MCF-7/6, IGF-I stimulated cell aggregation in absence but not in the presence of an anti-E-cadh antibody.

The direct visualization by immunofluorescence croscopy revealed that in MCF-7/IGF-IR cells, the I IR co-localizes with E-cadherin at the points of c cell contacts (Fig. 6). To our knowledge, this is the demonstration of co-localization of the IGF-IR wit cell–cell adhesion molecule. The interaction of the I IR with the E-cadherin complex was also confirme Western immunoblotting. Specifically, we found t in MCF-7 cells and MCF-7/IGF-IR clones, E-cadh

associated with IRS-1, SHC, and the IGF-IR. In MCF-7/IGF-IR cells, more IGF-IR appeared to be contained within E-cadherin complexes (Fig. 7). Interestingly, tyrosine phosphorylation of E-cadherin-associated SHC and IGF-IRs was not evidently increased in cells exposed to IGF-I for 48 h, whereas tyrosine phosphorylation of IRS-1 in the E-cadherin complex was quite prominent (Fig. 7). The molecular bases of this effect remain to be determined. It is possible that incorporation of the IGF-IR and SHC into adhesion complex accelerates dephosphorylation. IRS-1, resistant to this dephosphorylation, is probably a substrate of a different phosphatase(s).

In several experiments, we consistently observed an inhibitory effect of IGF-I on tyrosine phosphorylation of a 120-kDa protein whose position corresponded to that of E-cadherin (Fig. 7, MCF-7 cells, clone 12 and clone 15). Possibly, one of the functions of the IGF-IR in MCF-7 cells is to increase cell aggregation through dephosphorylation of E-cadherin. The interplay between growth factor- or oncoprotein-induced signaling and the regulation of cell–cell adhesion has already been described. For instance, EGF interferes with phosphorylation status of molecules engaged in adherens-type junctions, i.e., β-catenin is phosphorylated upon EGF stimulation [41]. In addition, expression of v-src causes tyrosine phosphorylation of E-cadherin and disrupts the cadherin–catenin complexes [42]. Cell–cell association can also be regulated by modification of the expression of adhesion proteins. For instance, overexpression of the ERB-B2 receptor or treatment with TGF-β inhibited expression of E-cadherin in normal mammary cells [43, 44]. In our model, there was no apparent modulation of E-cadherin expression.

Our experiments are still insufficient to resolve whether E-cadherin binds directly to either the IGF-IR or one of its substrates or if other intermediate proteins may be necessary to mediate this association. In fact, our preliminary data demonstrated that the IGF-IR is also present in β- and α-catenin precipitates. Similarly, since both IRS-1 and SHC can associate with the IGF-IR, binding of only one of those proteins to E-cadherin should be sufficient to form a multielement complex. Regardless of the nature of the association, the evident proximity of cell–cell adhesion molecules and the elements of IGF-IR signaling support our data implicating the IGF-IR in the regulation of epithelial aggregation.

The mechanism of IGF-IR-dependent cell–cell adhesion is currently unknown. The hypothesis that catalytic function of the receptor is involved is supported by the notion that in the presence of IGF-I, aggregation was induced and partial dephosphorylation of E-cadherin was observed. The other possibility is that the clusterization of receptors, due to activation by IGF-I or resulting from overexpression, induced concomitant clusterization of associated E-cadherin complexes, which created stronger contacts and promoted aggregation.

In summary, our findings suggest a complex role of the IGF-IR in breast cancer. On one hand, increased levels of IGF-IRs induce hypersensitivity to IGF-I in the presence of E2, which may provide a growth advantage for cancer cells under conditions of low IGF-I availability (for example, in patients undergoing tamoxifen treatment [45]). On the other hand, the overexpression of the IGF-IR inhibits cell scattering, and, therefore, may have a role in the growth of noninvasive, differentiated breast tumors. The latter supports the data demonstrating that in breast cancer, higher levels of receptor predict better prognosis [11]. The "antiscattering" function of the IGF-IR, however, does not seem to be universal. In other systems (such as invasive lung carcinoma), this receptor is required for metastatic activity [46]. Unquestionably, more studies are required to define the role of IGF signaling in metastasis as well as in other neoplastic processes.

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